11-2016

The Potential Antioxidant and Anti-Inflammatory Effects of Date Seed Powder in Rats

Fatima Theyab Saif Amer AI Meqbaali

United Arab Emirates University

College of Science

Department of Biology

THE POTENTIAL ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF DATE SEED POWDER IN RATS

Fatima Theyab Saif Amer Al Meqbaali

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences

Under the Supervision of Dr. Carine Platat

November 2016

Declaration of Original Work

I, Fatima Theyab Saif Amer Al Meqbaali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The Potential Antioxidant and Anti-inflammatory Effects of Date Seed Powder in Rats*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Carine Platat, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: _____ Date: 15 Dec 2016

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Carine Platat
   Title: Associate Professor
   Department of Nutrition & Health
   College of Food and Agriculture
   Signature: _____ Date: 24 November 2016

2) Member: Juma Musabah Mohammed Al- Kaabi
   Title: Associate Professor
   Department of Internal Medicine
   College of Medicine and Health Sciences
   Signature: _____ Date: 24/11/2016

3) Member (External Examiner): Rabih M. Kamleh
   Title: Vice President Research and Development
   Department of Quality Assurance Scientific and Regulatory Affairs
   Institution: Agthia Group, the UAE
   Signature: _____ Date: 24/11/16

This Master Thesis is accepted by:

Dean of the College of Science: Professor Ahmed Murad

Signature _____ Date 15/12/2016

Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

Signature _____ Date 15/12/2016

Copy 6 of 10

Abstract

Chronic diseases are among the main leading causes of mortality and morbidity in the United Arab Emirates (UAE). Oxidative stress (defined as an imbalance in the reactive oxygen species production/degradation ratio) in interaction with inflammation, has been highlighted as one major underlying mechanism in the development of these diseases. Due to their antioxidant and anti-inflammatory properties, polyphenols could prevent the development of chronic diseases. Interestingly, date seeds are particularly rich in such compounds. Nonetheless, an *in vivo* study aiming at studying and understanding antioxidant and anti-inflammatory effects of date seeds in serum and various organs is missing. Therefore, the present preclinical study is a step further to investigate these effects of date seeds in animal. Forty rats were fed for 13 weeks with either a control diet or a diet containing date seed powder (DSP) (0.2%, 0.4%, 0.8%). Biomarkers of the antioxidant status, protein and lipid oxidative damages, low-grade inflammation markers were measured in the serum and organs and histopathology was done. DSP was shown as not altering organs' function, as significantly increasing the antioxidant defense system in serum and organs and as decreasing protein and lipid oxidative damages in organs. Besides, DSP did not alter the immune inflammation markers. This highlights a preventive role of DSP against oxidative stress-related chronic diseases as well as suggesting its possible anti-inflammatory effects.

Keywords: Date seeds powder, chronic disease, antioxidants, oxidative damages, inflammation, polyphenols.

Title and Abstract (in Arabic)

التأثير المحتمل لمضادات الأكسدة والالتهاب في نوى التمر على الفئران

الملخص

تعتبر الأمراض المزمنة (chronic diseases) من بين الأسباب الرئيسية للأمراض والوفيات في دولة الإمارات العربية المتحدة. وقد تم تسليط الضوء على عملية الأكسدة (oxidative stress) (والتي تعرف بأنها خلل في التوازن بين عملية انتاج المشتقات التفاعلية للأكسجين وعملية تلاشيها) بالإضافة الى الالتهابات، كآلية أساسية واحدة في تطوير هذه الأمراض. ونظرا لخصائصها المضادة للأكسدة والمضادة للالتهابات، يمكن للبوليفينول (polyphenols) منع تطور هذه الأمراض المزمنة. ومن المثير للاهتمام، أن مسحوق نوى التمر (Date seed powder) غني بمثل هذه المركبات. وبما أنه لا توجد أي دراسات تهدف إلى دراسة وفهم التأثير المحتمل لمضادات الأكسدة والالتهاب في مسحوق نوى التمر على مصل الدم وأعضاء الفئران، فإن هذه الدراسة هي خطوة أخرى لدراسة تأثير مسحوق نوى التمر على الحيوانات. وقد تم تغذية أربعين فأرا لمدة 13 أسبوعا باستخدام نظام غذائي خال من مسحوق نوى التمر و نظام غذائي اخر يحتوي على مسحوق نوى التمر بنسب مختلفة (0.2%, 0.4%, 0.8%). ثم تم قياس المؤشرات الحيوية لنظام مضادات الأكسدة ومضادات الالتهاب، وقياس المنتجات البروتينية والدهنية المؤكسدة الناتجة عن عملية الأكسدة في الدم والأجهزة بالإضافة إلى التشريح المرضي. وقد أظهرت النتائج أن مسحوق نوى التمر لم يحدث أي تغيير في وظائف الأعضاء، كما أنه أدى إلى تحسين في نظام دفاع مضادات الأكسدة في الدم والأجهزة بالإضافة إلى خفض المنتجات البروتينية والدهنية الضارة الناتجة عن عملية الأكسدة في هذه الأعضاء. وكذلك لم يكون له أي تأثير على علامات الالتهاب. وعليه، فإن هذه النتائج تسلط الضوء على الدور الوقائي لمسحوق نوى التمر ضد الأمراض المزمنة المرتبطة بالتوتر الأكسدي بالإضافة الى الأهمية المحتملة له كمضاد للالتهابات.

مفاهيم البحث الرئيسية: مسحوق نوى التمر، الأمراض المزمنة، مضادات الأكسدة، أضرار الأكسدة، الالتهاب، البوليفينول.

Acknowledgements

I would like to thank my supervisor Dr. Carine Platat for her guidance, support, and assistance throughout my preparation of this thesis. I am especially grateful to Dr. Ayesha Al Dhaheri whose enthusiasm about and introduction to Environmental science got me started.

I would like to thank the chair and all members of the Department of Biology and the Department of Nutrition and Health at the United Arab Emirates University for assisting me all over my studies and research. My special thanks are extended to the Nutrition and Health Laboratories staff especially Mr. Usama Souka and Mrs. Serene Dominic for their assistance. My thanks go to the Library Research Desk for providing me with the relevant reference material.

Special thanks go to my parents, husband, brother, sisters and maid who helped me along the way. I am sure they suspected it was endless. In addition, special thanks are extended to the Al Meqbaali and Al Manei family for their assistance and friendship.

Dedication

To my husband, Mohammed who has been a constant source of support and encouragement during the challenges of graduate study and life

To my beloved mother for her constant, unconditional love and support

To the memory of my father Theyab AL Meqballi

To all of my family

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AD	Alzheimer's disease
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
CVD	Cardiovascular diseases
CRP	C- reactive protein
DSP	Date seed powder
GGT	Gamma-glutamyltransferase
GSH	Glutathione
HDL	High density lipoproteins
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
MDA	Malondialdehyde
ROS	Reactive oxygen species
TNFα	Tumor necrosis factors
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.1 Overview

Chronic diseases are the most common cause of death in the world today with 36 million people died in 2008, mainly from cardiovascular disease, cancer, chronic respiratory diseases and diabetes [1]. This represents an economic burden for many societies and the prevalence of chronic disease is still very worrying as they increase globally [2]. The UAE is particularly concerned since diabetes and cancer cause a high number of deaths in the country. The UAE ranks 16th worldwide for diabetes. According to the Diabetes Centre at Imperial College London 2015, 19.3% of the UAE population has diabetes. Meanwhile, cancer caused 13.9% of deaths in the Emirates in 2012. Lymphoid, hematopoietic and other related tissue cancers were the dominant cancers in Abu Dhabi. Recent findings also show that breast cancer leads to significant increases in mortality [3]. So far, no therapy or preventive approach has been able to prevent this worrying trend. As such, there is an urgent need to identify new strategies that can tackle these diseases.

It is well established that nutrition is a major contributing factor in chronic diseases. Dietary modification can have a very strong influence on health throughout your life [4; 5]. Diet influences the health of an individual and may determine whether, or not, they will develop diseases like cancer, cardiovascular disease or diabetes later in life. Thus, the beneficial nature of fruit and vegetables has been emphasized. There is strong evidence of the link between fruit and vegetable consumption and a reduced risk of cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts and age-related functional decline [5]. This is most

probably related to the nutritional properties of these foodstuffs as they are high in micronutrients, dietary fiber and phytochemicals such as polyphenols [6; 7].

Polyphenols are a complex family of molecules found commonly in plants. More than 800 different compounds have been identified. They can be divided into four groups that include flavonoids and phenolic acids. They have been demonstrated to display many different health properties. These include antioxidant and anti-inflammatory effects. This is of great importance since oxidative stress and inflammation are major underlying factors in developing chronic diseases and related health complications [8].

Oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense system. Due to the presence of pro-oxidants in the modern environment (chemicals, radiation, etc.), our bodies are constantly under pro-oxidant pressure, which increase the risk of chronic disease. Interestingly, it is not only through direct pathways that oxidative stress and inflammation are produced. They can, in the end, lead to chronic disease. It can also happen via other interactions. This creates complex networks, which can make a clear understanding of the sequence of the events that leads to chronic disease very difficult to arrive at [7]. The antioxidant and anti-inflammatory properties of polyphenols result in interesting potential in terms of the functional compounds that can prevent and/or treat chronic disease.

Date seeds, a local staple, have been identified as one of the best natural sources of polyphenols. They were used in the past to prepare a coffee-like beverage but nowadays they are mostly seen as a useless by-product of the date fruit. Date seeds contain high levels of different vitamins, minerals and fibers [9; 10; 11]. An

examination of the total polyphenols by ultra-high-performance liquid chromatography/ diode array detection/ electrospray ionization/ mass spectrometry (UPLC-DAD-ESI-MS) analyses estimated the date seed content of polyphenols to be close to 51 g kg^{-1}. Date seeds also contain Flavan-3-ols, in their monomeric and polymeric forms, (such as proanthocyanidins), with 46.800 g kg^{-1} of epicatechin and 3.380 g kg^{-1} of catechin [12]. Catechin and epicatechin are the polyphenols that display the greatest health effects [13]. Catechins and proanthocyanidins, in particular, have shown antioxidant capacities including a scavenging activity involving free radicals such as superoxide anions ($O\cdot_2^-$) a hydroxyl group (OH), nitric oxide(NO) and alkyl peroxyl radicals [14]. Catechins also have the ability to reduce the oxidative stress by modulating the enzymes that generate these free radicals, such as iNOS and XO [13]. Furthermore, in other studies, catechins have been shown to reduce lipid peroxidation and elevate endogenous antioxidants such as glutathione [15]. The antioxidant properties of polyphenols derived from date seeds have already been demonstrated *in vitro* [16].

These results were confirmed by studies conducted on animals where animal growth and recovery was demonstrated in cases of hepatotoxicity [17; 18; 19]. Date seed powder has also been studied in a short trial with male Wistar rats. After a 30-day diet containing date seed powder (7% and 14%), a reduction in liver and serum malondialdehyde (MDA), a marker of lipid oxidation, was clearly shown [16]. Because date seeds are particularly rich in polyphenols, the beneficial effects of date seeds in animals can be explained in terms of the role of polyphenols. The information about date seed powder's health benefits suggest it is a promising factor in the prevention and/or treatment of major nutrition-related chronic disease. Date seed powder has already been used to develop a healthy pita bread [20]. However,

even though the nutritional composition and *in vitro* antioxidant effect of date seeds powder has already been identified [9; 12; 16], its safety and the effects of antioxidants remain to be studied in a longitudinal study. In addition, although the key role of inflammation was identified in the development of chronic disease, there is no extant data related to the anti-inflammatory effects of date seed powder.

1.2 Statement of the Problem

Facing growth in nutrition-related diseases, many countries, including the UAE, have prepared strategies to tackle this issue. Oxidative stress and inflammation are two major mechanisms contributing to these diseases. Additionally, constant exposure to environmental pro-oxidants sources may also exacerbate the effects. Polyphenols are known to be plant compounds with well-defined antioxidant and anti-inflammatory properties. As such, they could well contribute to the prevention and/or treatment of chronic disease. Date seeds are a great source of polyphenols and safe to consume thus it can be reasonably assumed that date seed powder will have safe antioxidant and anti-inflammatory effects *in vivo*. This study aims to demonstrate just such a hypothesis.

Chapter 2: Relevant Literature

2.1 Oxidative Stress in Nutrition-related Chronic Disease

2.1.1 Definition

Oxidative stress can be defined as an imbalance between the metabolic generation of reactive oxygen/ nitrogen species (oxidants) and the ability of the organism's natural defense system to protect against the negative effects of antioxidant systems [21]. In a normal situation, cells are exposed to pro-oxidants from both endogenous and exogenous sources, but are equipped with antioxidant defense systems that neutralize the harmful effects to the cell (Figure 1). Reactive oxygen and nitrogen species include superoxide, hydrogen peroxide, hydroxyl radical, lipid hydro peroxides, peroxynitrite and other related species that are normally formed as a by- product of the aerobic metabolism process, and also play an important role in cell signaling and hemostasis [22].

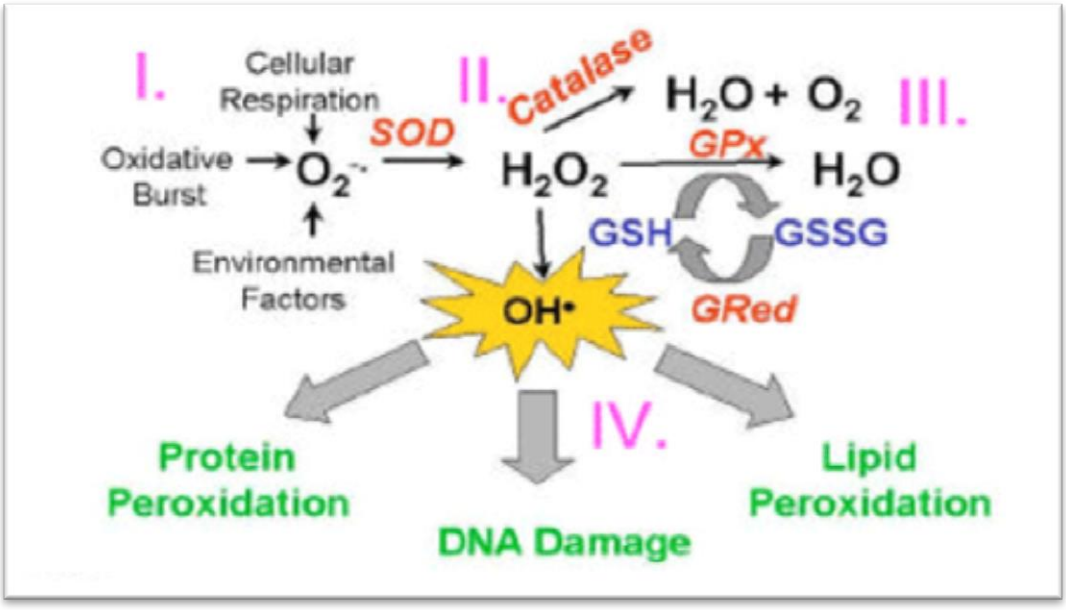

Figure 1: Biological Action of Free Radicals [23]

2.1.2 How Oxidative Stress Can Lead to Chronic Disease

The reactive oxygen and nitrogen species produced in the body can cause oxidative damage in various substrates of the body (Figure1) including lipids, proteins and nucleic acids. The oxidation of these substrates contributes to the development of chronic diseases that include cancer, cardiovascular disease, diabetes, other age-related diseases and perhaps even to the aging process itself [24, 25]. Some studies have shown that oxidation of low density lipoprotein (LDL) can be a causative agent in cardiovascular disease, and that the oxidation of DNA can contribute to the development of human carcinogenesis [26]. In addition, the increase of oxidative stress in adipose tissue is an important mechanism in obesity-associated metabolic syndrome. The production of ROS is specifically increased in adipose tissue when it is associated with an increase in NADPH oxidase expressions and a decrease in antioxidative enzyme expression [27].

2.1.2.1 Lipid Oxidation

Lipids, especially polyunsaturated fatty acid, are more likely to be oxidized. Malondialdehyde (MDA) is the main product of polyunsaturated fatty acid oxidation and one of the lipid peroxidation biomarkers [28, 29]. MDA exhibits high reactivity with biomolecules such as proteins, DNA and phospholipids to form adducts that result in bio molecular damage [29]. MDA-DNA adducts may lead to mutations, cell cycle arrest, strand breaks and the induction of apoptosis. This MDA-induced DNA alteration may contribute significantly to altering the properties of biomolecules, leading ultimately to chronic disease [30, 31]. Clinical studies showed that the reaction between MDA and proteins is a major cause of coronary heart disease and strokes. Besides this, MDA levels in the plasma of patients with Diabetes Mellitus

has been shown to increase and MDA was also found in the atherosclerotic plaques promoted by diabetes [32].

2.1.2.2 Protein Oxidation

Proteins are also prone to oxidization by ROS, which leads to the formation of protein-protein cross-linkages and the oxidation of the protein backbone, itself a product of protein fragmentation [33]. Protein carbonyl is the most important protein oxidation marker [33, 34, 35, 36]. An accumulation of protein carbonyls characterizes many human diseases including diabetes, inflammatory bowel disease (IBD), Alzheimer's disease (AD), Parkinson's disease, essential hypertension, cystic fibrosis, ulcerative colitis and arthritis [36].

2.1.2.3 DNA Oxidation

DNA is another molecule that can be damaged by oxidative stress and can cause oxidative DNA lesions that can lead to mutations. Although the body has different mechanisms to protect DNA such as antioxidants and/or enzymes that repair DNA, oxidative stress can still result in cancer. DNA oxidative damage can cause modifications in DNA structure including DNA-protein cross-links, base-free sites, base and sugar lesions and strand breaks [37].

2.1.3 The Role of Antioxidants

Antioxidants are either naturally synthesized inside the living organism, or externally supplied through food and other supplements [38]. Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing damage caused by free radicals. They can, therefore, enhance the immune defense system and lower the risk of cancer and other degenerative diseases (Figure 2). The

antioxidant defense system includes enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [39], which act via a series of reactions to convert reactive oxygen species (ROS) to more stable molecules, such as water and oxygen (O_2). Additionally, small molecular weight, non-enzymatic antioxidants (e.g., GSH, NADPH, thioredoxin, vitamins E and C and trace metals such as selenium) function as direct scavengers of ROS.

These enzymatic and non-enzymatic antioxidant systems are important in maintaining the intracellular redox balance and in minimizing the cellular damage caused by ROS [38]. Exogenous antioxidants are mainly dietary antioxidants. These include micronutrients such as vitamin C (L-ascorbic acid), vitamin E, or beta-carotene, and polyphenols [39]. Beta-carotene and vitamins E and C have been closely studied and strong antioxidant activity has been shown [26; 40]. Polyphenols are a complex family of molecules, often found in plants, that include flavonoids and phenolic acids that can control the harmful effects of oxidative stress [26].

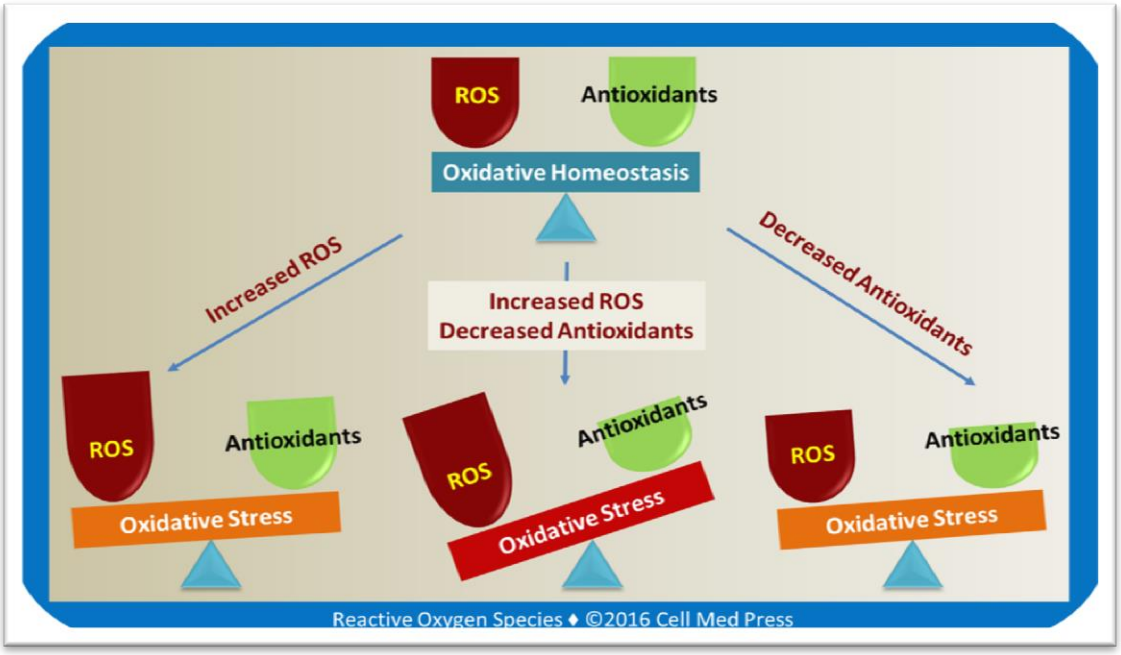

Figure 2: Oxidative Homeostasis [41]

2.1.4 Conclusion

Oxidative stress occurs when the equilibrium between the oxidant (ROS/RNS) and the antioxidant is broken. The balance between oxidant and antioxidant is critical in maintaining a healthy biological system. Oxidative damage can result from the actions of ROS/RNS and/or a weak antioxidant system. Many body molecules can be affected, including lipids, proteins and DNA. This oxidative damage contributes to the pathogenic processes in many diseases. They can be reduced by antioxidant systems such as endogenous and exogenous compounds acting as free radical scavengers.

2.2 Inflammation: Another Critical Pathway Leading to Chronic Disease

Over the last decade, the critical role played by inflammation in the development of chronic disease has been highlighted. There is also growing evidence that certain metabolic conditions, like high insulin levels, are linked to certain nutritional practices that promote inflammation and ultimately the development of chronic diseases such as obesity, diabetes, cardiovascular disease (CVD) and cancer.

2.2.1 Inflammation: A Definition

Inflammation is the immune response of body tissue to an injury or infection and can be either acute or chronic in scale. Acute inflammation is a short-lived physiologic response to injury or infection and can last from a few minutes to a few days. It is characterized by an increased blood flow, greater blood vessel permeability and the accumulation of white blood cells leading to swelling, heat, redness and pain at the affected site.

These physical symptoms induce the generation of new cells and a synthesis of the collagen matrix that stimulates the healing process in the damaged tissue. When acute inflammation fails to arrest the infection, or heal the injury, chronic inflammation is created. Chronic inflammation is a long-term physiologic response to many factors such as microbial or viral infections, stress, toxins, poor nutrition and the aging process, it may last for weeks, months or even years. Ultimately, chronic inflammation is a failure of the body's immune system to maintain a healthy homeostatic state [42].

2.2.2 Role of Cytokines

2.2.2.1 Cytokines: A Definition

Cytokine is a general term used for a large and diverse family of small non-antibody proteins and peptides generated by a wide range of cells types, which act as regulators under both normal and pathological conditions in order to modulate the functional activities of individual cells and tissues. It also regulates communication between cells. They have a central role in the inflammatory process as they exert anti- and pro- inflammatory effects. Cytokines are different from hormones. Hormones are the primary product of a specific tissue, or cell, whereas cytokines are produced by most cells. More importantly, on a molar basis, cytokines are far more potent than hormones as they act at lower molar concentrations than most hormones [43]. For example, the concentration of cytokine IL-1 that induces gene expression and a synthesis of cyclo-oxygenase-1 (COX-2) to synthesize inflammatory mediators (called prostaglandins and thromboxanes) is 10 pM. The ability of IL-12 to induce IFNγ is 20 pM [44].

2.2.2.2 Cytokine Types

About 30 cytokines are recognized. These include the interleukins IL-1 to IL-24, tumor necrosis factors (TNFs), and transforming growth factors (TGF, Beta 1–3) (Figure 3). In general, cytokines have a low molecular weight (200 amino acids) and have specific receptors. Actually, cytokine is a general name but it is still the best descriptor, as most cells are able to produce and respond to these proteins. Other labels include monokine, a cytokine made by monocytes; lymphokine, which is cytokine made by lymphocytes; chemokine, cytokines with chemotactic activity and interleukin cytokines which are made by one leukocyte acting on other leukocytes.

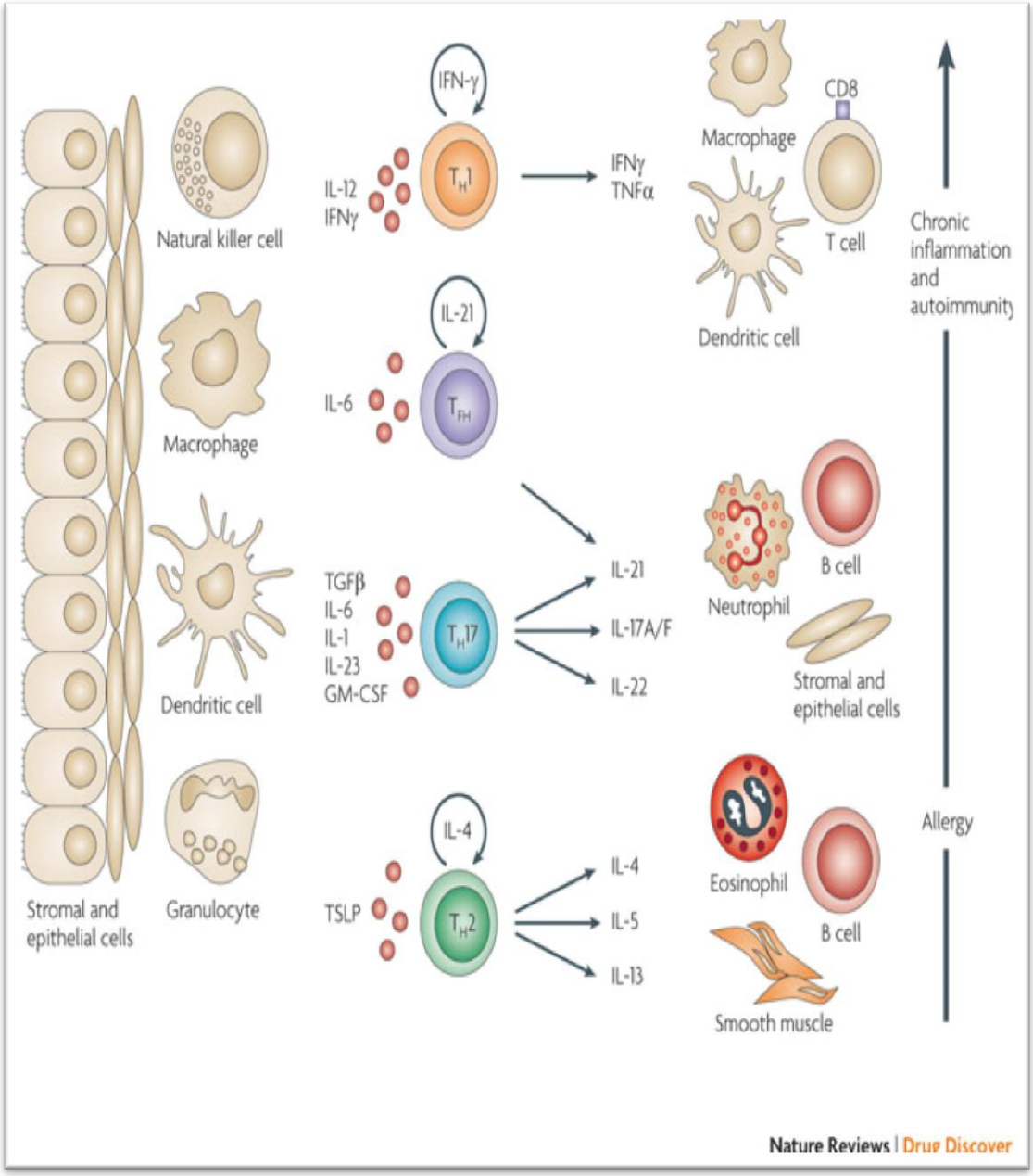

Figure 3: Pro-inflammatory Cytokines [45]

2.2.2.3 Cytokine Action

Some cytokines display an autocrine action meaning they act on the cells that secrete them. Others can be described as having a paracrine action where they act on nearby cells or have endocrine effect on more distant cells (Figure 4). Cytokines are usually produced in a cascade-like effect as one cytokine stimulates target cells in

order to create additional cytokines. Some cytokines act as growth factors, while others are pro-inflammatory and some more can suppress the pro-inflammatory action. These are anti-inflammatory molecules, as compared to some cytokines, which polarize the immune response to antigens. For example, IL-4, IL10 and IL-13 are effective activators for B-lymphocytes and, in addition, they have a potent role as anti-inflammatory agents that can suppress the genes in pro-inflammatory cytokines such as IL-1, TNF and some chemokines.

Another example is IFN-^{g} which is similar to IFN-α and IFN-β and provides antiviral protection. IFN-^{g} is also an activator in cytotoxic T cells. However, IFN-^{g} is considered to be a pro-inflammatory cytokine because of its ability to increase TNF activity and prompt nitric oxide (NO) [46]. Pro-inflammatory cytokines are mostly produced by activated macrophages and are involved in the regulation of inflammatory reactions [47]. The early release of cytokines determines the nature of the inflammatory response, which can either induce protective immunity or be harmful by driving immunopathology. Cytokines combined with an environment that includes other cytokines or specific tissues can lead to a differentiation in cell types, which can trigger the production of other cytokine combinations that either clear invading pathogens or induce inflammatory disorders. Therefore, these early cytokines are one of the key steps in the development of disease as has been emphasized in the research.

Tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), IL-6, IL-12 and IL-23 and granulocyte macrophage colony-stimulating factors (GM-CSF) are among the most critical inflammatory cytokines. IL-1 and TNFα are inducers of endothelial adhesion molecules, which are important in the adhesion of leukocytes to the

endothelial surface, before migrating to the tissues. All of these cytokines have major effects on multiple parts of the immune system and they mediate inflammation in gene products that are not usually produced in a healthy person. Cytokines IL-1, TNFα and, in some cases, IFN-ᵍ are mainly effective in simulating the expression of these genes and they also initiate a cascade of inflammatory mediators by targeting the endothelium. This happens whether it is induced by infection, ischemia, trauma, toxins or immune-activated T cells. Chemokines are small peptides (8000 d) and represent another class of pro-inflammatory cytokines that can assist leukocytes to pass from circulation into tissues. IL-8 is a typical example. It also activates neutrophils that can cause tissue damage [45; 46].

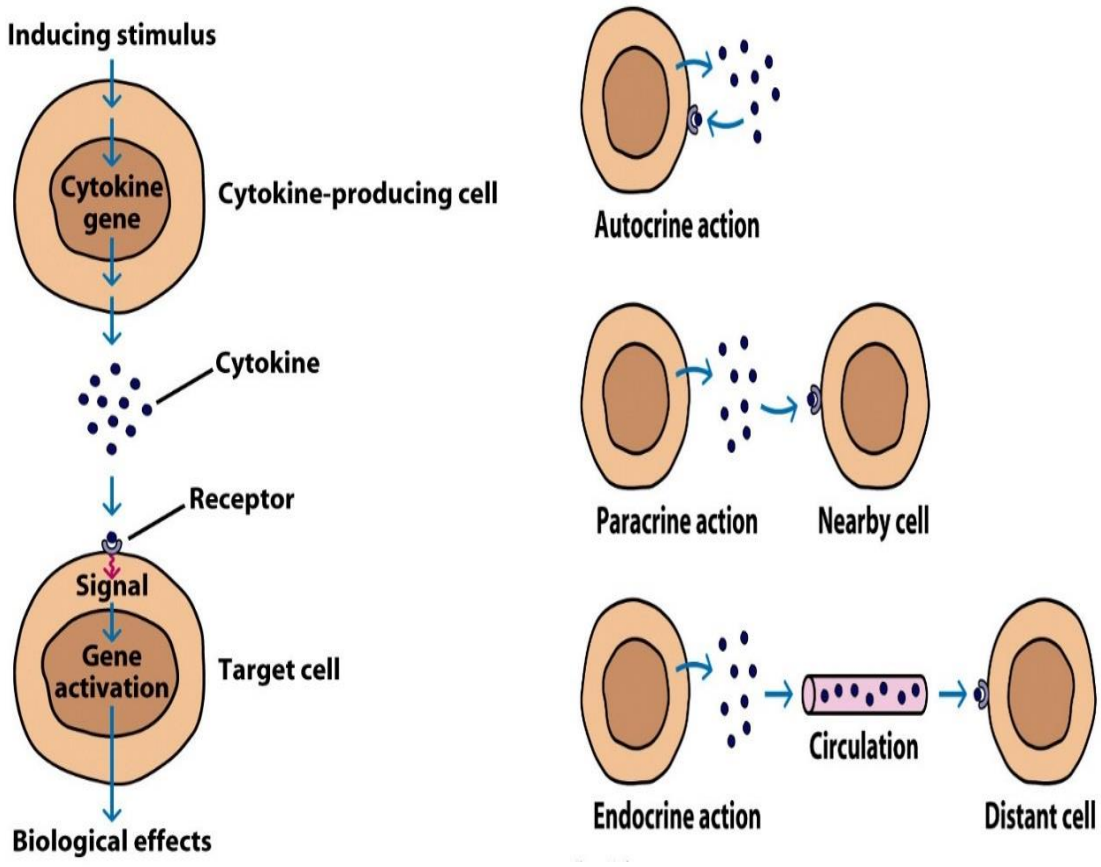

Figure 4: Cytokine Action [48]

2.2.3 Low-grade Inflammation and Chronic Disease

Low-grade inflammation is an immune system response that is characterized by a two to fourfold rise in the level of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and TNFα and also in acute phase reactants such as C-reactive protein (CRP), fibrinogen, sialic acid, haptoglobin and serum amyloid A. It is also characterized by low albumin concentrations, which are synthesized in the liver and regulated by cytokines such as IL-6 and TNF-alpha. Although these increases are far from the levels observed during the acute phase, severe infections and low-grade inflammation are associated with the development of many chronic diseases (Figure 5).

Chronic low-grade inflammation is increasingly recognised as a pathological feature of numerous common chronic diseases. One of the most important and sensitive systematic markers of low-grade inflammation is CRP which is a classic sensitive acute phase reactant in normal conditions and also present in low concentrations. CRP is synthesized by hepatocytes in response to pro-inflammatory cytokines, especially IL-6. It has the ability to indicate inflammation at an early stage as it rises in the serum within 24 hours. In addition, its long plasma half-life (12-18 hours) is constant under most conditions. Therefore, the sole determinant for circulating CRP is the rate of synthesis. This, in turn, directly reflects the intensity of the pathological process stimulating CRP production. This property is useful for the early detection of patients who are at risk for inflammatory disease.

CRP is both a marker and a mediator of atherosclerosis and coronary heart disease as it contributes to many aspects of atherogenesis including lipid uptake by macrophages, activating the complement pathway, releasing pro-inflammatory

cytokines, promoting endothelial dysfunction, inducing the expression of tissue factors in monocytes and the inhibition of nitric oxide production. Studies show that CRP levels usually increase in response to a wide variety of infections, inflammation and cancerous conditions. According to the Center for Disease Control and Prevention and the American Heart Association, a CRP level of >3 mg/L indicates a higher risk of CVD, whereas CRP levels of 1-3 mg/L suggest a medium risk. CRP levels of <1 mg/L suggest a lower risk [49]. In addition, evidence has shown that individuals with CRP levels of > 3 mg/L have a four to six times greater risk of developing diabetes than individuals with lower levels of CRP. One aspect of the link between heart disease and diabetes is inflammation which can result in obesity because the adipocytes produce messenger proteins that induce the production of CRP [50].

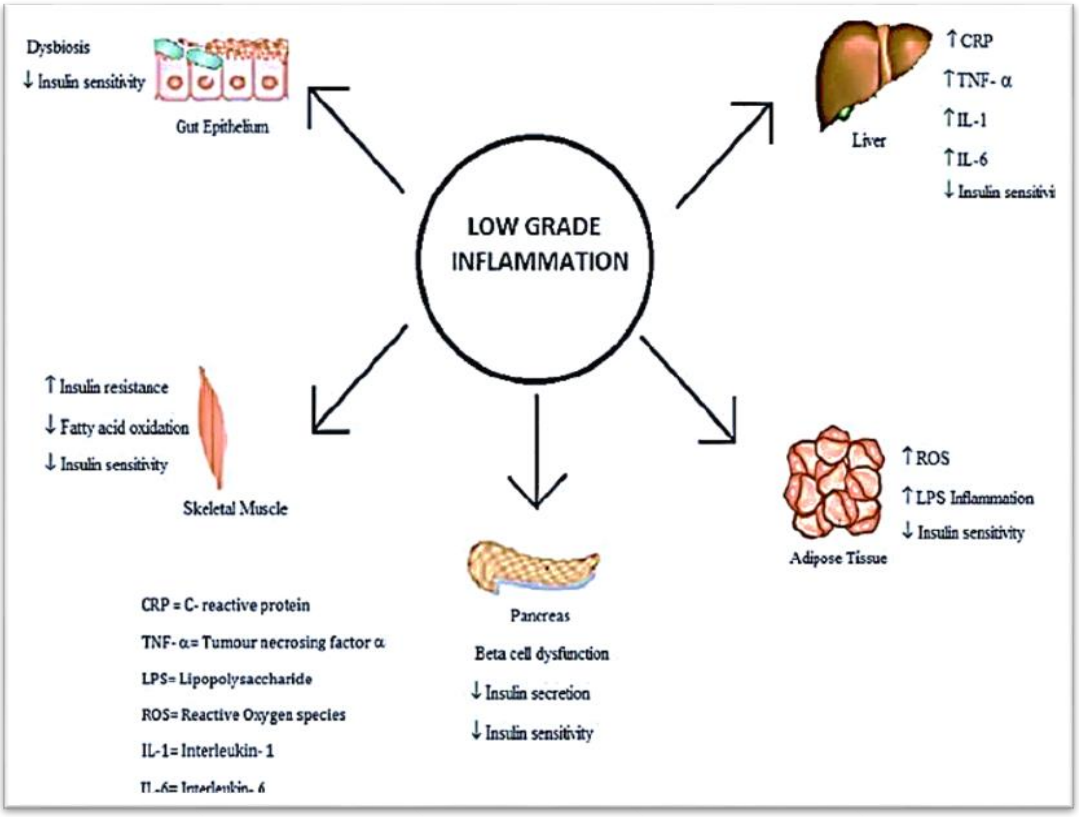

Figure 5: Low-grade Inflammation Impacting Various Organs [51]

2.2.4 The Challenge of Detecting Low-grade Inflammation

The critical role of low-grade inflammation in the development of chronic disease is clear, thus making early detection particularly important. Nevertheless, as above, although low-grade inflammation is characterized by a two to fourfold rise in the level of pro-inflammatory cytokines, this level still remains far from the levels observed during acute or severe infections [52]. Therefore, new and more effective techniques for detecting low concentrations of low-grade inflammation circulation cytokines are emerging. Recently, cytokine and chemokine research has become an important tool as a diagnostic, prognostic and therapeutic agent in dealing with and treating human diseases. They play a crucial role in achieving a deeper understanding of immunology, inflammation, allergic reactions, cancers and atherosclerosis [53].

There are several methods for detecting and analyzing cytokines: traditional ELISA assays, enzyme-linked immunosorbent spot (ELIspot) assays, antibody array assays and bead-based assays. To identify specific cytokines involved in any inflammatory or immune response, it is necessary to screen panels of cytokines. This often requires a level of automation and/or high throughput, as well as high sensitivity in order to measure low concentrations of low-grade inflammation cytokines. As such, magnetic beads can make the process of automation and higher throughput screening easier thanks to features such as walk-away washing. Bead-based assays are cost-efficient because they allow for multiplexing, i.e. the simultaneous detection of multiple targets. Multiplexing allows for smaller sample volumes and higher throughput automation. Compared to a typical ELISA, which require 100 to 200 microliters for each sample, Bio-Plex assays, using Luminex micro-beads require just 12.5 microliters, or less, per reaction. In bead-based assays,

immune detection does not occur on the flat surface of the membrane or microliter plate, but on micron-sized spheres. Each bead contains a unique blend of fluorophores that act as a signature and are associated with a single analyte-bead identifier. 1 corresponds to IL-2, identifier 2 to IL-4 and so on. Multiplexing is accomplished by combining different bead sets with their associated capture antibodies in one master mix and incubating that mix with each sample in a microliter plate [54].

2.2.5 Conclusion

Inflammation is the immune response of body tissues to injury or infection and can be either acute or chronic. Cytokine is a general term used for a large and diverse family of non-antibody small proteins and peptides. Some cytokines act as growth factors, others are pro-inflammatory and some suppress pro-inflammatory activity and are called anti-inflammatory molecules. Tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), IL-6, IL-12 and IL-23 and granulocyte macrophage colony stimulating factors (GM-CSF) are among the most critical inflammatory cytokines. Low-grade inflammation is an immune system response characterized by a two to fourfold rise in the levels of pro-inflammatory cytokines. Low-grade inflammation is associated with the development of many chronic diseases, including Alzheimer's disease, cancer, cardiovascular disease and diabetes**.** Therefore, the detection of low-grade inflammation is crucial and can be achieved via several different methods that detect and analyze cytokines. These methods include traditional ELISA assays, enzyme-linked immunosorbent spot (ELIspot) assays, antibody array assays and bead-based assays.

2.3 Inflammation and Oxidative Stress Interactions

Free radicals are both a cause and a result of inflammation. Damage caused by free radicals can lead to inflammation and, in turn, chronic inflammation can induce the production of more free radicals, which consequently create more inflammation. This is a vicious cycle, which can damage many systems in the body. On one hand, at low concentrations, the production of ROS is essential for innate immune response regulation and is involved in regulating the immune system when signaling injury. Additionally, redox signaling is important in the signaling pathways engaged by various inflammatory conditions.

The production of ROS can regulate activation of redox-regulated transcription factors (NF-κB and AP-1) as well as cytokine production. On the other hand, when ROS are produced in excessive amounts, they can react with various other biomolecules, thus altering their structure and function and resulting in cellular damage and chronic inflammatory disease [55]. The response of the immune system to an attack by endogenous and/or exogenous antigens is to recruit mast cells and leukocytes to the damaged site. This leads to a respiratory burst where oxygen uptake increases and consequently increases ROS production and accumulation at the damaged site. This, in turn, induces signaling cascades that prompt the production of pro-inflammatory cytokines and chemokines.

Alternatively, the inflammatory cells themselves can also produce soluble mediators such as cytokines and chemokines that induce greater production of ROS. These key soluble mediators can activate signal transduction cascades in addition to inducing changes in transcription factors such as nuclear factor kappa B (NF-κB), signal transducers and activators of transcription 3 (STAT3), activator protein-1 (AP-

1), hypoxia-inducible factor-1α (HIF1-α), the nuclear factors of activated T cells (NFAT) and NF-E2 related factor-2 (Nrf2), which can mediate immediate cellular stress responses. This induces cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Abnormal expressions of inflammatory cytokines including TNF-α, IL-1, IL-6 and chemokines have also been reported to play a role in oxidative stress-induced inflammation [56].

2.4 The Inflammation-oxidative Stress Cycle and Chronic Disease

The inflammation-oxidative stress cycle can cause damage to many bodily systems but can also mediate most chronic diseases including cardiovascular disease, cancer, diabetes and obesity through a variety of mechanisms.

2.4.1 Cardiovascular Disease

Evidence to support the oxidative stress and inflammatory hypothesis behind cardiovascular disease is found in the elevation of acute-phase reactant C-reactive proteins (CRP) and also in the role of oxidative stress in atherosclerosis, particularly via the oxidative modification of low-density lipoproteins (LDL). The oxidized LDL and it is components play a significant role in activating the innate immune system by interacting with Toll-like receptors. This interaction causes an intracellular signaling cascade that induces the expression of a wide range of pro-inflammatory molecules, including cytokines, chemokines, eicosanoids, proteases, ROS, reactive nitrogen species and stimulatory molecules.

Endothelial dysfunction is a common target and has been accepted as an early determinant in the development of atherosclerosis. Oxidative stress can cause injuries to different types of cells such as endothelium cells resulting in endothelial

dysfunction. This dysfunction stimulates a pro-inflammatory environment as evidenced by the increased endothelial expressions of adhesion molecules and the imbalance of arachidonic acid metabolites and chemo-attractant molecules. By forming a positive feedback loop, vascular inflammation leads to endothelial dysfunction which is associated with increased ROS production.The overall pro-inflammatory, pro-oxidant micro-environment disrupts vascular function, mainly through decreasing the bioavailability of vasodilator nitric oxide (NO) and maintaining conditions of oxidative stress through the excess generation of ROS and RNS [57].

2.4.2 Cancer

Studies have proved a relationship between inflammation, oxidative stress and cancer. It has been reported that one-quarter of all cancer cases worldwide are related to chronic inflammation. Continued active inflammation responses induce ROS overproduction from inflammatory cells and leads to cell damage or cellular hyperplasia. During inflammation, ROS can interact with DNA in mitotic cells causing permanent genomic mutations such as gene deletion, point mutation or gene rearrangement. In addition, more free radicals will be produced as a response to the inflammation in order to activate the genes involved in DNA repair. In cases of chronic inflammation, the cellular antioxidant system decays and this increases the rate of ROS production that induces the DNA. Chronic inflammation prompts cells to transform due to the incidence of frequent damage to the DNA from inflammatory cells causing a higher frequency of mutation. Chronic inflammation also increases the prevalence of growth-supporting stimuli and growth factor production, such as vascular endothelial growth factors (VEGF) [58].

2.4.3 Type 2 Diabetes

Oxi-flammation (oxidative stress and inflammation) has a critical impact on insulin resistance and type 2 diabetes. Increased inflammation, oxidative stress, glucotoxicity and dyslipidemia cause a greater workload for β-cells and the β-cells reach a point where they are no longer able to meet the ever-increasing demand for insulin. This results in diabetes. Recent studies have shown that chronic low-grade inflammation and the activation of the innate immune system are both involved in the pathogenesis of type 2 diabetes. However, the exact mechanism by which inflammatory cytokines affect the glucose metabolism in humans is still not clear. Insulin is known to act as an inhibitor in CRP synthesis, which increases insulin deficiency and indicates that there might be a positive correlation to type 2 diabetes, whereby cytokine-induced insulin resistance enhances acute phase response [59].

2.4.4 Obesity

One of chronic diseases is obesity. It develops due to the interaction of many factors including social, behavioral, psychological, metabolic, cellular and molecular factors. Obesity is characterized by an increase in body weight that may induce systemic oxidative stress that, in turn, induces the irregular production of adipokines. Alternatively, excessive adipose tissue can increase oxidative stress because adipocytes are a source of pro-inflammatory cytokines, including TNF-α, IL-1 and IL-6. These cytokines stimulate the production of ROS/RNS through macrophages and monocytes; therefore, obesity is considered as a state of chronic inflammation. Excessive accumulation of fat can produce cytokines such as TNF-α which generates ROS in tissues due to the cellular damage caused by pressure from the fat cells. As a result the lipid peroxidation rate will increase [60]. The increased concentrations of

TNF-α and IL-6 are associated with obesity and can cause many pathological complications including restricting the action of the insulin by suppressing insulin signal transduction. This might interfere with the anti-inflammatory effect of insulin and so promote inflammation [61].

2.4.5 Alzheimer's Disease (AD)

Evidence suggests that inflammation contributes significantly to the pathogenesis of Alzheimer's disease (AD). Inflammation occurs in pathologically weak regions of the brain and is associated with an increased expression of pro-inflammatory cytokines and acute phase proteins, which do not usually appear in a normal brain. Inflammatory cells produce different inflammatory mediators such as pro-inflammatory cytokines, chemokines, monocyte chemo-attractant proteins, thromboxanes, leukotrienes, prostaglandins, macrophage inflammatory proteins, coagulation factors, complement factors, reactive oxygen, nitric oxide species and C-reactive proteins. Studies report that levels of IL-1α, IL-1β, IL-6 and TNF-α are increased in AD-affected brain tissue. These cytokines secrete other proteins onto senile plaques. In addition, synergistic effects can also occur between cytokines such as IFN-γ and β-amyloid that enhance the production of TNF-α and reactive nitrogen species that are also toxic to neurons. The production of pro-inflammatory mediators may interact on multiple levels with neurodegeneration. Therefore, pro-inflammatory cytokines can not only cause cell death but can also affect classical neurodegenerative pathways [62].

2.4.6 Conclusion

Free radicals are both the cause and a result of inflammation. Damage caused by free radicals can lead to inflammation and, in turn, chronic inflammation can produce more free radicals and thus create even more inflammation. This vicious cycle damages many bodily systems and can also mediate many chronic diseases such as cancer, diabetes, cardiovascular disease and obesity via various mechanisms.

2.5 Polyphenols: Antioxidant and Anti-inflammatory Plant Compounds

2.5.1 Definition

Polyphenols are naturally occurring products found in fruit, vegetables, cereals and beverages and are secondary metabolites in plants. Polyphenols contribute to the color, flavor, odor, bitterness, astringency and oxidative stability of food. There are more than 8,000 polyphenolic compounds found in various plant species. Polyphenols can be classified into many groups (Figure 6). The main classes include phenolic acids, flavonoids, curcuminoids and stilbenes. They differ in their number of phenol rings and structural elements that bind these rings together. The differences in polyphenol types are in their primary aromatic rings, oxidation status and functional groups [63].

Flavonoids are made of two aromatic rings bound together by three carbon atoms to form an oxygenated heterocycle. They can be classified into six subclasses based on the function of the heterocycle. This includes flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. Favonoids are the most closely studied group of polyphenols.

Phenolic acids come in two main derivatives: one from benzoic acid and the other from cinnamic acid, where hydroxycinnamic acids are more common than hydroxybenzoic acids and consist chiefly of p-coumaric, caffeic, ferulic and sinapic acids.

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. The presence of Stilbenes in human food is very low and found mainly in

grapes. In fact, these compounds are produced in response to an infection and act as antifungal phytoalexins.

Lignans are another type of polyphenol containing a 2, 3-dibenzylbutane structure formed by the dimerization of two cinnamic acid residues. One of the richest sources of lignan is linseed.

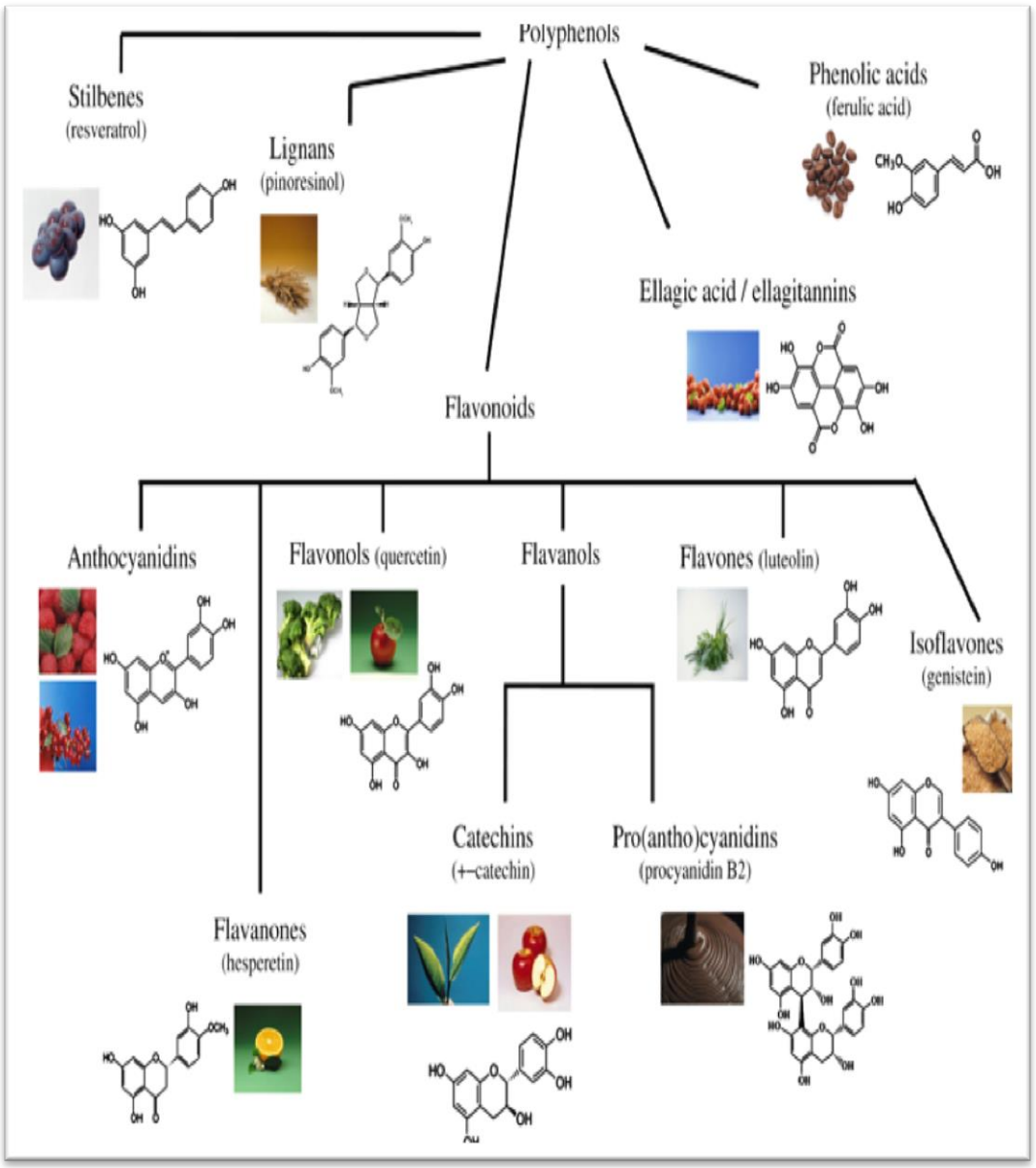

Figure 6: Dietary Polyphenol Subclasses and their Best Sources [64]

2.5.2 Plant Sources for Polyphenols

The distribution of polyphenols in plant tissue is not uniform, as the insoluble part of the phenolic acid is located in the cell wall, while soluble phenolics are found in plant cell vacuoles. Outer layers of plants contain higher levels of phenolic acids as compared to their inner parts. The polyphenol content of plants is affected by different factors including ripeness, environmental factors, processing and storage [65]. The richest source of polyphenol are spices including cloves and star anise. Additionally, dried herbs such as peppermint and Mexican oregano contain high concentrations of flavanones. Cocoa powder and chocolate are also rich in polyphenols, mainly catechins and proanthocyanidins. In addition, berries have a high concentration of anthocyanins, especially the dark colored ones such as black chokeberries, black elderberries, blueberries and blackcurrant. Several seeds and nuts are also among the richest sources of polyphenols. Flaxseed is rich in lignan secoisolariciresinol; chestnuts and walnuts are rich in ellagitannins; hazelnut, pecan nuts and almonds are rich in proanthocyanidins, while soy flour and roasted soybeans are rich in isoflavones [66].

2.5.3 Bioavailability of Polyphenols

Bioavailability is usually defined as the portion of an ingested nutrient that reaches the systemic circulation of the organism, which is the specific site where it exerts its biological effect. Polyphenols affect health due to many factors including absorption, distribution, metabolism and elimination. In addition, the bioavailability of each polyphenol type varies, as there is no relation between the number of polyphenols in food and their bioavailability in the human body. The most common types of polyphenol in the human diet are not necessarily the most active in the

human because they have a lower intrinsic activity due to being poorly absorbed from the intestine, highly metabolized or rapidly eliminated [67]. It is the chemical structure of polyphenols, not its concentration, that determines the absorption rate and extent as well as the nature of the metabolites circulating in the plasma [26].

The bioavailability of polyphenolic compounds is determined by measuring the metabolite concentration in the plasma and urine after the compound has been ingested. The absorption process in polyphenols is followed by an extensive process of conjugation and metabolizing in the intestinal cells mediated by hebetic enzymes. The potential toxicity of polyphenols is controlled by the liver where it is prepared for either biliary or urinary excretion by increasing hydrophobicity. The conjugation process in polyphenols mainly includes glucuronidation, sulfation and methylation processes [67].

2.5.4 The Health Benefits of Polyphenols

2.5.4.1 Antioxidant Effects

A considerable body of literature supports the role of oxidative stress in the pathogenesis of chronic disease and the role of polyphenols in their prevention. The preventative role of polyphenols is related to their antioxidant effect. Polyphenols' antioxidant role is to protect cell components from oxidative damage caused by the scavenging of free radicals. Further, polyphenols interact with cells directly through cell receptors and enzymes that can modify the redox status of cells and improve the status of oxidative stress biomarkers. The antioxidant effect of polyphenols can improve cell survival rates by inducing apoptosis and also prevent tumor growth [8]. For example, flavan-3-ols, including catechin and epicatechin, were identified as the polyphenols with potentially the greatest beneficial health effects [12] (Figure 7).

Catechins and proanthocyanidins, in particular, have been widely studied for their antioxidant capacities and have demonstrated a scavenging effect on various forms of free radicals including superoxide anions ($O\cdot_2^-$), the hydroxyl group (OH), nitric oxide (NO) and alkyl peroxyl radicals [14]. Also, catechins have the ability to reduce oxidative stress by modulating the enzymes that generate free radicals such as iNOS and XO [13].

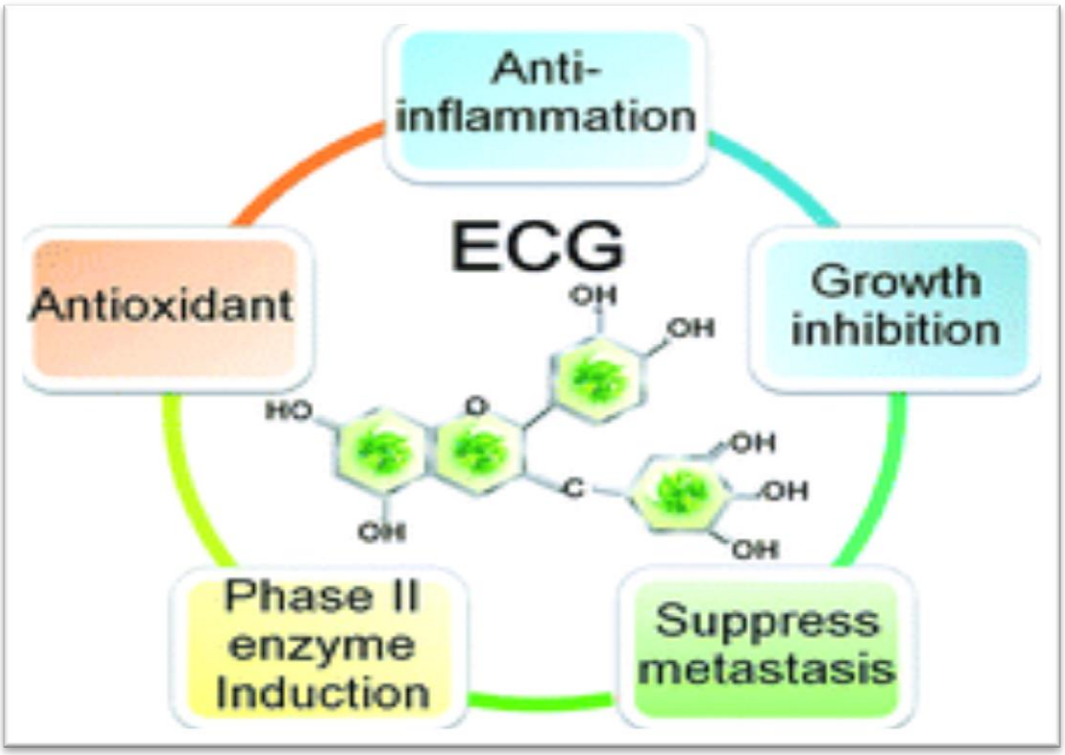

Figure 7: Epicatechin Health Benefits [68]

2.5.4.2 Anti-inflammatory Effects

Studies have shown that polyphenols have significant anti-inflammatory effects in *vivo* and *in vitro* not only as antioxidants but also as modulators of inflammatory redox signaling pathways [69; 65]. Polyphenols exert an anti-inflammatory effect *in vivo* via several different mechanisms. These include an inhibition of eicosanoids that generate enzymes such as phospholipase A2 and cyclooxygenase, nitric oxide inhibitors, cytokines and NF-κB inhibitors. As far as

cyclooxygenase is concerned, polyphenols can control its activity as well as its gene expression in different cell types [70].

Experiments suggest that polyphenols can inhibit nitric oxide (NO) production by suppressing nitric oxide synthase (NOS) enzyme expression and NOS activity. NO has the capacity to interact with a variety of enzymes, thereby altering their function and influencing inflammatory reactions. Another important mechanism is the ability of polyphenols to control cytokines, which are major mediators of local and intercellular communication in immune and inflammatory processes. Many polyphenolic flavonoids have been reported to inhibit the expression of pro-inflammatory cytokines. For example, quercetin and kaempferol have been shown to inhibit TNF-α and also IL-6 production during inflammation. On the other hand, the inhibitory action of cytokines excreted by polyphenolic flavonoids is combined, on occasion, with anti-inflammatory cytokines [69].

In addition, another useful strategy for the treatment of the inflammation is the ability of polyphenols to inhibit NF-κB, which plays a critical role in the immune, inflammatory, stress, proliferative and apoptotic responses by controlling special transcription factors for NF-κB [65]. In various studies carried out in different countries [71], it has been found that a dietary pattern characterized by high levels of polyphenols in vegetables, fruits and legumes is inversely associated with blood inflammation markers such as CRP, IL-6 and adhesion factors. The anti-inflammatory action of polyphenols appears to be compound and tissue specific. For example, quercetin flavonols inhibit the release of TNF-α while green tea polyphenols inhibit IL-8 production in human nasal fibroblasts. Also quercetin and

kaempferol flavonols inhibit gene expression and the secretion of TNF-α, IL-1b or IL-6 in some cells [72].

2.5.5 Polyphenols and Chronic Disease

Epidemiological studies have repeatedly shown an inverse association between the risk of chronic human disease and the consumption of a polyphenol rich diet. This is mainly due to anti-oxidation and the anti-inflammatory effects of the polyphenols [73]. The preventive and curative effects of polyphenols diseases have been extensively studied and they have been shown to exhibit antioxidant and anti-inflammatory properties.

As well as these properties, dietary polyphenols impact on transcriptional networks and signaling cascades that modulate gene expression and promote anti-inflammatory properties. Polyphenols involved in the inhibition of nuclear factor kappa beta (NF-kB) can regulate the inflammatory process in many chronic diseases including cardiovascular disease, diabetes, cancer and Alzheimer's disease. They do so by modulating the expression of the pro-inflammatory genes. The efficient transcriptional activation of NF-kB depends on the phosphorylation of active subunit P65. Polyphenols may block P65 phosphorylation, rendering NF-kB transcriptionally inactive [74]. Studies have shown that the incidence of coronary heart disease is limited by the consumption of polyphenols, as they work to inhibit LDL oxidation: a key mechanism in atherosclerosis [26; 75]. In addition, some polyphenols, specifically quercetin, inhibit enzymes such as cyclooxygenase (COX) and lipooxygenase (LPO) that are associated with the release of interleukins and chemokines.

It is widely accepted that a diet rich in polyphenols can prevent various types of cancer. Dietary polyphenols may exert their anticancer and chemoprevention effects through different mechanisms such as removal of carcinogenic agents, controlling cancer cell signaling and antioxidant enzymatic activities. They can also stimulate apoptosis and cell cycle arrest [76]. Polyphenols interfere with each stage of the carcinogenesis process including initiation, promotion and progression. Additionally, polyphenols effect the metabolism of pro-carcinogens by controlling the expression of cytochrome P450 enzymes that can activate carcinogens. The anti-cancer effects of polyphenols are related to their antioxidant properties that protect DNA from oxidative damage by carcinogens. They have also been shown to inhibit cyclooxygenase, hydroperoxidase, protein kinase C, focal adhesion kinase, NFκB and cell cycle regulators [26].

An increasing number of epidemiological studies have concluded that a diet rich in polyphenols may lower the risk of developing diabetes as it affects digestion and the absorption of dietary carbohydrate. Polyphenols can contribute to the inhibition of glucose absorption in the intestinal tract and its uptake by peripheral tissues. In addition, polyphenols show a potent ability to inhibit the enzymes responsible for the digestion of dietary carbohydrates, changing then to glucose: e.g. α-glycosidase and α-amylase. Furthermore, some polyphenols are able to regulate key pathways in the carbohydrate metabolism and hepatic glucose homeostasis such as glycolysis, glucose oxidation, glycogenesis and the glycogen content of the liver. Gluconeogenesis and the glucose output of the liver are usually impaired in the case of diabetes. Polyphenols can improve β cell function and insulin behavior by protecting β-cells from hyperglycemia-induced, oxidative-induced damage and by

modulating key cellular signaling pathways. All these protective mechanisms improve glucose homeostasis and insulin resistance [77].

Indeed, polyphenols not only have a direct effect on pancreatic β-cells and stimulating the secretion of insulin through the activation of specific cellular targets, but also, protects these cells from damage caused by oxidative stress and inflammation. Both of which are typically elevated during diabetes [78]. Moreover, consumption of foods rich in bioactive anti-inflammatory compounds, such as polyphenols, has been reported to decrease the inflammation associated with obesity due to their anti-inflammatory and anti-oxidant properties.

Some studies suggest that the anti-obesity effects of polyphenol-rich diets may be related to the ability of polyphenols to interact, directly or indirectly, with adipose tissues via different pathways [79]. These pathways include the suppression of fat absorption, the inhibition of angiogenesis in adipose tissue, the inhibition of the differentiation of pre-adipocytes to adipocytes, the stimulation of apoptosis of mature adipocytes and a reduction in the chronic inflammation associated with obesity through the regulation of adipokines production [80]. Adipokines are a variety of proteins secreted by adipocytes and they exhibit important metabolic and inflammatory properties: these include leptin, adiponectin and visfatin [81].

2.5.6 Conclusion

Polyphenols are a large family of naturally occurring products that are distributed widely in plants. Principal sources of polyphenols include fruit and beverages such as tea, red wine and coffee. Polyphenol bioavailability is determined by chemical structure not concentration. Epidemiological studies have repeatedly

shown an inverse association between the risk of chronic human diseases, including cardiovascular disease, cancer, diabetes and obesity and the consumption of a polyphenol rich diet. This can be attributed to polyphenols having diverse health benefits that include both antioxidant and anti-inflammatory properties.

2.6 Date Seeds: A Promising Functional Ingredient to Prevent/ Treat Chronic Disease

2.6.1 Date Seeds

Phoenix dactylifera is a palm from the Phoenix genus. It is cultivated in many countries for its sweet, edible fruit and is considered as the most important tree in most Arab countries [11]. Egypt is among the top five date-producing countries, along with Iran, Saudi Arabia, Iraq and Pakistan. According to FAO statistics in 2014, dates are the second most abundant crop in United Arab Emirates (UAE), with production reaching up to 548,135.75 tons.

Therefore, large quantities of date seeds are available as a by-product of the date production process. Unfortunately, they are often considered to be as waste product and have previously been used in a limited way as animal feed or in the making of caffeine-free, coffee-flavored drinks [9]. Developing new applications for date seeds will economically support date producing countries.

2.6.2 Nutritional Composition

The fruit of the date palm is composed of a fleshy pericarp and a seed. The date seeds represent about 15% of the total weight of the date fruit [9]. The date seed contains different chemical compounds such as saturated fatty acids like stearic and

palmitic acid, and unsaturated fatty acids such as linoleic and oleic acids, which inhibit the 5-α reductase enzyme. They also contain zinc (Zn), cadmium (Cd), calcium (Ca) and potassium (K) [82]. Date seeds contain 3.1–7.1% moisture, 2.3–6.4% proteins, 5.0–13.2 fats, 0.9–1.8% ash and 22.5–80.2% dietary fiber. Also, date seeds contain high levels of phenolic acid (3102– 4430 mg gallic acid equivalents/ 100 g), antioxidants and dietary fiber (78–80 g/100 g) [83].

2.6.3 The Biological Effects of Date Seeds: What Do We Know?

Date seed powder is also used in some traditional medicines and has been investigated for its potential health benefits [84]. It is sometimes included in animal feed to enhance growth and the testosterone levels in blood plasma [18]. In addition, date seed extract displays an ability to restore the normal functional status of a diseased liver and to protect against any subsequent carbon tetrachloride hepatotoxicity in rat livers [85]. Date seeds contain high levels of antioxidants and their effect on oxidative damage and antioxidant status *in vivo* has been researched. One study conducted in male Wistar rats revealed that after a 30-day period of a diet containing date seeds (7% and 14%), a reduction in liver and serum malondialdehyde (MDA) was detected [16]. Another study evaluated the antioxidant and anti-inflammatory activities of methanolic and water extracts from the edible portions of date fruit (Phoenix Dactylifera L., Family Palmae), and also assessed the methanolic extract of date seeds in relation to adjuvant arthritis in rats: a model of chronic inflammation. The results revealed that oral administration of methanolic extract from date seeds produced a significant reduction in swelling on the foot by up to 35.5%. Additionally, the antioxidant status (plasma, vitamin C, E and A and β-

carotene) increased significantly on administration of different extracts, while the plasma level of MDA reduced by a significant factor [86].

2.6.4 Conclusion

Date seeds are a major local foodstuff. They also represent a promising functional food product thanks to their nutritional composition and high polyphenol content. It's *in vitro* antioxidant effect has been isolated and the anti-inflammatory properties can be identified. However, these latter effects have not been properly investigated so far. In addition, the biological effects associated with date seeds have yet to be investigated and established *in vivo*.

Chapter 3: Methods

3.1 Diets and Feeding Regimen

3.1.1 Date Seeds

Date seeds from the Khalas variety were obtained from Al Ain Dates Factory (Al Ain, UAE). The season (summer) of collecting tamr (fully ripe dates) is usually spread over a period of 2–3 months. Samples were collected randomly from tamr batches at the end of the season, with no preference to size, color, appearance or firmness. The seeds were first soaked in water, washed to get rid of any adhering date flesh, air-dried, and ground into coarse powder using a hammer mill.

3.1.2 Animals

The protocol used in this study was reviewed by the Animal Research Ethics Committee, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain and was conducted according to the Declaration of Helsinki principles. It was recorded as protocol number A19-12 and was approved on June, the 4th, 2012. The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" [87]. Normal Wistar male rats (43-113 g) were obtained from the College of Medicine and Health Sciences Animal Facility, UAE University, Al Ain. The rats were housed in plastic cages under controlled conditions of 12-h light/12-h dark cycle, 50% humidity and 25±3ºC.

3.1.3 Experimental Design

The animals were randomly divided into four groups. They were fed for 13 weeks before sacrifice. An isocaloric and isonitrogenous basal diet, similar to the

American Institute of Nutrition AIN-93G purified rodent diet [88] was used. Three Date seed powder diets, DSP1, DSP2 and DSP3, were prepared by using 0.2%, 0.4% and 0.8% DSP in the diet respectively. During the experimental period, the control group (5 rats) received the basal diet, DSP1, DSP2 and DSP3 groups (9 rats each) received DSP1, DSP2 and DSP3 diets, respectively. Water and feed were provided ad libitum to the rats. The doses for rats which were considered here were determined based on 1)the recommended amount of carbohydrates for adult (210g per day), 2) The average food intake of rats 25g per day, according to data from a previous study [16]. and the initial animal weight (200g).

3.1.4 Preparation of Serum, Plasma, Tissue Fragments and Homogenate

At the end of the experimental period, rats were anaesthetized with pentobarbital and killed following blood withdrawal via heart puncture. Blood samples were drawn into dry (for obtaining serum) and heparinized (for obtaining whole blood) tubes. Portions of serum were immediately separated after centrifuging the blood sample and used for measuring the levels of glucose, total cholesterol and HDL, total protein, albumin, lactate dehydrogenase (LDH), urea, creatinine, creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), vitamin C, lipid peroxidation product Malondialdehyde (MDA) and protein-bound carbonyls. Liver, muscle, heart and brain were removed, blotted and weighed and 200 g/ kg homogenate were prepared in ice-cold 15.5 g/L KCL in 0.05 mol/L Tris buffer (pH 7.4) using a homogenizer. Portions of organs homogenate were processed for measuring the levels of protein, vitamin C, vitamin E, Glutathione (GSH), MDA and protein-bound carbonyls.

3.2 Biochemical parameters

Glucose, total cholesterol, HDL, LDH, urea, creatinine, CK, AST, ALT, ALP and GGT were measured in serum by using enzymatic colorimetric methods on Roche/Hitachi Cobas c systems (Integra 400 Plus) [89].

3.2.1 Glucose

Glucose is the major carbohydrate present in the peripheral blood, oxidation of glucose is the major source of cellular energy in the body. The test principle is UV test, enzymatic reference method with hexokinase. Glucose is phosphorylated with adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase (HK). The product, glucose-6-phosphate (G6P) is then oxidized with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) to NADH in the reaction catalyzed by glucose-6-phosphate-dehygrogenase (G6PDH). The formation of NADH causes an increase in absorbance at 340 nm. The increase was directly proportional to the amount of glucose in the sample [89].

3.2.2 Cholesterol

Cholesterol is a steroid with a secondary hydroxyl group in the C3 position, it synthesized in many types of tissue but particularly in the liver and intestinal wall. The test principle of the enzymatic, colorimetric method is based on the cholesterol esters which are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide formed can be used to oxidize an indicator in the presence of POD. In this test, H2O2 oxidizes 4 aminoantipyrine and phenol to a quinoneimine dye in the presence of peroxidase

(POD). The color intensity of the dye formed is directly proportional to the cholesterol concentration. It was determined by measuring the increase in absorbance at 512 nm [89].

3.2.3 Low Density Lipoproteins (LDL)

Low density lipoproteins play a key role in causing and influencing the progression of atherosclerosis and, in particular, coronary sclerosis. The LDLs are derived from VLDLs (very low density lipoproteins) rich in triglycerides by the action of various lipolytic enzymes and are synthesized in the liver. Homogeneous enzymatic colorimetric assay was used to measure LDL. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantipyrine and HSDA to form a purple-blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and was measured photometrically [89].

3.2.4 High Density Lipoprotein (HDL)

High density lipoprotein is responsible for the reverse transport of cholesterol from the peripheral cells to the liver. The test principle is based on the homogeneous enzymatic colorimetric assay. In the presence of magnesium ions and dextran sulfate, water-soluble complexes with LDL, VLDL, and chylomicrons are formed which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approximately 40%). Cholesterol

esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. The color intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It was determined by measuring the increase in absorbance at 583 nm [89].

3.2.5 Urea

Urea is the major end product of protein nitrogen metabolism. It is synthesized by the urea cycle in the liver from ammonia which is produced by amino acid deamination. Urea is excreted mostly by the kidneys but minimal amounts are also excreted in sweat and degraded in the intestines by bacterial action. Determination of blood urea nitrogen is the most widely used screening test for renal function. The test principle is based on the Kinetic test with urease and glutamate dehydrogenase. Urea is hydrolyzed by urease to form ammonium and carbonate. In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed. The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It was determined by measuring the absorbance at 340 nm [89].

3.2.6 Creatinine

The test principle of this kinetic colorimetric assay is based on the Jaffé method. In alkaline solution, creatinine forms a yellow-red complex with picrate.

The rate of dye formation is proportional to the creatinine concentration in the specimen. To correct for non-specific reaction caused by serum/plasma pseudo-creatinine chromogens, including proteins and ketones, the results for serum or plasma are corrected by -18 μmol/L (-0.2 mg/dL) [89, 90].

3.3.7 Lactate Dehydrogenase

The lactate dehydrogenase (LDH) enzyme is widely distributed in tissue, particularly in heart, liver, muscles and kidneys. Elevated serum levels of LDH have been observed in a variety of diseases. the test principle is based on the UV assay where the Lactate dehydrogenase catalyzes the conversion of L-lactate t pyruvate; NAD is reduced to NADH. The initial rate of the NADH formation id directly proportional to the catalytic LDH activity it is determined by photometric measuring the increase in absorbance [89].

3.2.8 Creatinine Kinase

The CK enzyme is a dimer composed of subunits derived from either muscle (M) or brain (B). Three isoenzymes have been identified: MM, MB, and BB. Normal serum CK is predominantly the CK-MM isoenzyme. Elevated CK-serum levels are found in skeletal muscle disease, particularly muscular dystrophy. The test principle is based on the method according to the recommendations of the International Federation of Clinical Chemistry (IFCC), the Société Française de Biologie Clinique (SFBC), the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (SCE), and the Deutsche Gesellschaft für Klinische Chemie (DGKC). The rate of the NADPH formation is directly

proportional to the catalytic CK activity. It was determined by measuring the increase in absorbance at 340 nm [89].

3.2.9 Aspartate Aminotransferase (AST)

The enzyme aspartate aminotransferase (AST) is widely distributed in tissue, principally hepatic, cardiac, muscle, and kidney. Elevated serum levels are found in diseases involving these tissues. Hepatobiliary diseases, such as cirrhosis, metastatic carcinoma, and viral hepatitis also increase serum AST levels. Method according to the International Federation of Clinical Chemistry (IFCC), but without pyridoxal-5'-phosphate. AST in the sample catalyzes the transfer of an amino group between L-aspartate and 2-oxoglutarate to form oxaloacetate and L-glutamate. The oxaloacetate then reacts with NADH, in the presence of malate dehydrogenase (MDH), to form NAD+. The rate of the NADH oxidation is directly proportional to the catalytic AST activity. It was determined by measuring the decrease in absorbance at 340 nm [89].

3.2.10 Alanine Aminotransferase (ALT)

The enzyme alanine aminotransferase (ALT) has been widely reported as present in a variety of tissues. The major source of ALT is the liver, which has led to the measurement of ALT activity for the diagnosis of hepatic diseases. Elevated serum ALT is found in hepatitis, cirrhosis, obstructive jaundice, carcinoma of the liver, and chronic alcohol abuse. The test principle is based on the method according to the International Federation of Clinical Chemistry (IFCC), but without pyridoxal-5´-phosphate. ALT catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and NAD+. The rate of the NADH oxidation

is directly proportional to the catalytic ALT activity. It was determined by measuring the decrease in absorbance at 340 nm [89].

3.2.11 Alkaline Phosphatase (ALP)

Alkaline phosphatase in serum consists of four structural genotypes: the liver-bone-kidney type, the intestinal type, the placental type and the variant from the germ cells. It occurs in osteoblasts, hepatocytes, leukocytes, the kidneys, spleen, placenta, prostate and the small intestine. The ALP test is based on colorimetric assay in accordance with a standardized method in the presence of magnesium and zinc ions, p-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol. The p-nitrophenol released is directly proportional to the catalytic ALP activity. It was determined by measuring the increase in absorbance at 409 nm [89].

3.2.12 Gamma-glutamyltransferase (GGT)

Gamma-glutamyltransferase is used in the diagnosis and monitoring of hepatobiliary diseases. Enzymatic activity of GGT is often the only parameter with increased values when testing for such diseases, and is one of the most sensitive indicators known. The test principle is based on enzymatic colorimetric assay Gamma-glutamyltransferase transfers the γ-glutamyl group of L-γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The amount of 5-amino-2-nitrobenzoate liberated is proportional to the GGT activity in the sample. It was determined by measuring the increase in absorbance at 409 nm [89].

3.2.13 Total Protein

Total protein content was measured in blood and organs by following the method of Lowry et al. [91]. The principle of Lowry’s method lies in reactivity of the

peptide nitrogen with copper (II) ions in the reagent under alkaline conditions. Subsequently Folin's ciocalteu reagent is reduced by copper catalyzed oxidation of aromatic acids to give a blue color which can be measured at 750nm. This method is sensitive to low concentrations of protein (0.05-2mg of protein per ml). The concentration of sample is extrapolated from a standard curve build with known concentrations of purified protein such as Bovine Serum Albumin. A critical point in Lowry's procedure is that a variety of compounds can interfere with the assay. These include certain buffers, drugs, lipids, sugars, salts, nucleic acids, sulphydryl reagents, ammonium ions, zwitter ionic buffers, thiol compounds etc. Such compounds should be removed or diluted before running Lowry's assay.

The Chemicals were used in this experiment are Bovine Serum Albumin (lyophilized powder), Sodium carbonate Na_2CO_3, Sodium hydroxide NaOH, Copper sulphate pentahydrate $CuSO_4.5H_2O$, Potassium sodium tartarate $KNaC_4H_4O_6.4H_2O$, Folin's Ciocalteu reagent and PBS tablets or (Sodium chloride NaCl, Potassium chloride KCl, Disodium hydrogen phosphate Na_2HPO_4, Potassium dihydrogen phosphate KH_2PO_4 for Reagent A was prepared by dissolving 1% sodium carbonate in 0.1 N NaOH. Reagent B was prepared by dissolving 0.5 % CuSO4 in 1% sodium potassium tartarate. Reagent C was prepared from Reagent A and Reagent B by mixing it in a ratio of 50:1. 1mL of this solution was added to 100 µL of the sample and was incubated for ten minutes. 100 µL of Folin's Reagent was added and incubated in dark for half an hour. Absorbance at 750 nm was taken using UV-Visible Spectrophotometer. The results were extrapolated from the standard curve obtained and the unit is mg/ml.

3.3 Antioxidant Status Biomarkers

3.3.1 Vitamin C

Vitamin C concentration was measured by Omaye et al. method [92], after reaction with 2,4-dinitrophenylhydrazine at 520nm. The method is based on the oxidation of Ascorbic acid by copper to form dehydro ascorbic acid and diketo gulonic acid. These products react with 2, 4, dinitrophenyl hydrazine (DNPH) to form a complex, which in the presence of sulfuric acid undergoes rearrangement to form a product with absorbance at 515nm. The reaction was carried out in the presence of thiourea to provide a reducing medium to prevent interference from non-ascorbic acid chromogen. Chemicals and reagents were used in this experiment are 2,4-DNPH (2,4-Dinitrophenylhydrazine) reagent, 5%Thiourea solution, 0.6%Copper Sulfate, 65%H2SO4, 5% TCA, 5%metaphosphoric Acid in 10% TCA solution and Ascorbic acid. 0.1ml of serum was mixed with 0.4 ml 5% ice-cold TCA to precipitate the proteins, then centrifuged at 3000 rpm for 15 minutes. 0.3ml of the supernatant was collected and 0.125ml of DTC (DNPH-thiourea-copper sulphate reagents in ratio 20:1;1 which prepared fresh daily for the work) was added to it. The mixture was incubated at 60°C for one hour and then cool in Ice for 10 minutes. 0.5 ml cold 65%H2SO4 was added to each tube and mixed. Tubes were kept to stand at room temperature for 30 minutes and reading was taken at 515nm. The results are extrapolated from the standard curve obtained and the unit is ug/ml.

3.3.2 Vitamin E

Vitamin E was measured by HPLC using a fluorescence detector with excitation at 205 nm and emission at 340 nm [93]. Vitamin E Standard was prepared

from vitamin E (alpha- Tocopherol) standard which is a 99% oily stock from Sigma. Working stock of the standard was prepared with concentration 20mg/ml, 2000 mg of vitamin E standard, then it was added to methanol and shacked to be dissolved. Volume was made up to 100ml in a standard volumetric flask. The stock standard was diluted to provide working standards with different concentration (1, 3, 5, 10, 20 mg/ml) for the assay. All the flasks were kept away from light and heat. Stored at 4°C and the working standards was filtered using a 0.45 µm membrane syringe filter into UPLC sample vials (amber). 500µl of the sample was taken and added to 1000µl of ethanol. Then it was vortex well and 4 ml of Hexane was added. The mixture was vortex well and then Centrifuged at 3000 rpm for 5 - 10 minutes. After that 3000µl of the Hexane layer (supernatant) was aliquot to a fresh tube. The Hexane was evaporated using pressurized Nitrogen gas till dryness. For the analysis, the residue was dissolved with 500µl Methanol and filtered using 0.45µm syringe filter onto HPLC sample vial (amber). Finally, the samples were injected in the HPLC.

The Liquid Chromatographic analysis was carried out using the ACQUITY UPLC System (Waters, Milford, MA, USA) with the PDA eλ detector (Photo Diode Array detector), Acquity Sample Manager, Binary Solvent Manager and the total system was controlled with Waters Empower Software. The analytical column was ACQUITY BEH C18, 1.7 µm stationary phase particle size reversed phase column (2.1 mm I.D. X 100 mm length), Waters USA. The mobile phase consisted of Solvent A: Water: Acetonitrile (90:10), Solvent B: Acetonitrile: Methanol (50:50). Flow rate was 0.7 ml / min, column temperature was35 °C, injection volume was 10 µl and the detection wavelengths was 291 nm for Vitamin E and 285 nm for Vitamin E acetate.

3.3.3 Glutathione

Glutathione was measured spectrophotometrically at 412 nm after reaction with dithionitrobenzoic acid. This assay is based on the measurement of the non-protein sulfhydryl groups (NP-SH) by spectroscopy using the method as described by Ellman, who used 5, 5'-dithiobis-(2-nitrobenzoic acid) DTNB for the measurement. In the reaction DTNB is reduced by –SH groups to form 2-nitro-5mercaptobenzoic acid. The product formed has an intense yellow color and can be used to measure the –SH groups, reading the solution at wavelength 412nm. The Chemicals were used in this experiment are DTNB, Methanol, Tris salt, EDTA and TCA. All procedure in the experiment was carried out at room temperature. 100µl of homogenate is mixed with 100µl of 20% TCA. The mixture was vortex and incubated in ice for 20 minutes. The sample was centrifuged at 10,000×g for 15 minutes and 100µl of the sample supernatant in to 96 well plate. 100µl of 10% TCA was added to one well which is assigned as blank. 200µl of Tris buffer followed by 5µl of DTNB solution was added to the blank and sample wells in the microplate. Incubate for 5 minutes in an agitator shaker. Read the absorbance at 412 nm. The results were calculated using Beer-Lamberts law using the molar extinction co-efficient. The unit used to express the data is nmol /g tissue

$$Concentration\ (Initial) = \left(\frac{Absorbance}{13.6\ \times 0.96945}\right) \times 305$$

Where,

$13.6 mM^{-1}cm^{-1}$ is the molar extinction co-efficient of reduced DTNB (TNB)

0.96945cm is the path-length for 305µl in NUNC edge 96 well plate which is calculated using the formula,

$$Pathelength = \frac{(4 \times Volume\ in\ well)}{(\prod \times Diameter^2)}$$

Concentration Initial corresponds to the concentration of NP-SH in the supernatant taken to be assayed in the 96-well plate. The final concentration in nmol/g tissue is calculated by,

$$Concentration\ (Final) = \left(\frac{Concentration\ Initial \times 2 \times 10}{0.1}\right)$$

Where, 2 is the dilution factor in step 1 of the protocol (yields the NP-SH content in 100µl of the initial sample). 10 is the conversion factor to yield the NP-SH content in 1ml of the homogenate. 0.1 is used to calculate the NP-SH in 1g of the tissue (Since homogenate was prepared as 10%, 1ml of homogenate corresponds to 0.1g of the tissue) [94].

3.4 Oxidative Damage Biomarkers

3.4.1 MDA

The concentration of the lipid peroxidation product MDA was measured by using the modified procedure of Li and Chow [95]. The reaction mixture was extracted with isobutanol and the fluorescence intensity was measured with excitation at 515 nm and emission at 550 nm using a spectrofluorometer. 1,1,3,3 Tetramethoxypropane was used as standard. MDA standard is a 99% pure liquid stock from Sigma. For preparation of standard the following calculation is followed.

Density of the MDA Std = 0.997g/ml (997g/L)

Mass % = 99% = 0.99

Molar mass = 164.20g/mol

$$No.of\ moles = \frac{Mass\ \times Mass\%}{Molar\ Mass}$$

$$No.of\ moles\ = \frac{997 \times 0.99}{164.20} = 6.011\ moles$$

$$Molarity = \frac{Moles}{litre} = \frac{6.011}{1} = 6.011M$$

Hence the standard is a 6.011M solution.

Conversion of units: 6.011moles/1000ml = 0.006011moles/ml

0.006011moles/ml = 0.006011×109nmol/ml =6011×103nmol/ml

To prepare a working stock of the standard with concentration 1000nmol/ml, 16.6µl of the MDA Std. is added to 0.2N HCl and volume is made up to 100ml in a standard flask. Dilutions of this working stock provides working standards with different concentration (1, 5,10,20,40 nmol/ml) for the assay. Standard were prepared by adding 1 ml of the TBA solution to 1 ml of the working standard, then vortex well and incubated for 1 hour at 60ºC. 500 µl of the mixture was transferred to new tube and 500µl of butanol was added to it. The mixture was vortex well and the tube was kept to stand for a minute. Once the phase in mixture has separated, the upper layer has been carefully removed using the syringe. Filter the 0.45µm filter onto HPLC sample vial (amber). The standards were injected.

For the sample, 400µl of the sample was taken and added to 400µl of ice cold 20% TCA solution. The samples were vortex well and incubated in ice for 20 minutes. The samples were centrifuged at 11,000rpm for 15 minutes and aliquoted 400µl of the supernatant to a fresh tube. 400µl of TBA solution was added to the supernatant, vortex well and incubated at 60C for 1 hour. After incubation, the samples allowed to cool down a little. 800µl of butanol was added to the sample and vortex well. Then the mixture was stand for phase separation and once the phase in mixture has been separated, the upper layer was carefully removed using the syringe. Finally, the samples were filtered using 0.45µm filter onto HPLC sample vial (amber) and injected. For the HPLC Conditions, the High-Performance Liquid Chromatographic analysis was carried out using a Waters Breeze HPLC system (Waters Corporation, USA): 1525 Pump, 2475 Multi λ Fluorescence detector, 717 Auto sampler and system controller with a PC control program. The analytical column is XTerra MS C18, 5 µm pore size reversed phase column (4.6 *150 mm), Waters USA. The mobile phase consisted of Methanol – 0.05 M KH_2PO_4 buffer

PH=6.8 (40:60, v/v) containing 0.2 %(v/v) Tri ethanol amine. HPLC was isocratic a condition at flow rate 1 ml/min. Column temperature 35 °C. Fluorescence detector wavelength was set at 532 nm (excitation) and 553 nm (emission) and the injection volume was 20 µl.

3.4.2 Protein-bound Carbonyls

The content of protein-bound carbonyls, which is used to assess the extent of protein oxidation, was determined spectrophotometrically at 530nm by the 2,4 dinitrophenylhydrazine method of Levine et al [96]. The method used for the assay of protein carbonyl in biological samples relies on the reaction between protein carbonyl and 2, 4-dinitrophenylhydrazine (DNPH). DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone. The aliphatic hydrazones thus formed can be measured by spectroscopy between 360-380nm. The Chemicals were used in this experiment are 2, 4-dinitrophenylhydrazine (DNPH), Hydrochloric Acid (HCl), Guanidine HCl, Ethanol, Ethyl acetate, Tricholoro acetic acid (TCA), Disodium hydrogen phosphate (Na2HPO4) and Sodium dihydrogen phosphate (NaH2PO4). Before proceeding, make sure all reagents have thawed down to room temperature in case it was refrigerated.

For protein carbonyl assay, each sample is assayed twice – control and test respectively. Prepare tubes for all samples and label them appropriately. Aliquot 100µl of the sample to control and test tubes respectively. 400µl of 2.5M HCl was added to the sample in control tube and 400µl of 0.2% DNPH to the sample in test tubes. The tubes were vortex thoroughly and incubated at room temperature under dark conditions for 1 hour with intermittent vortex every 10 minutes. 500µl ice-cold 20% TCA was added to both tubes of the sample, vortex well and incubated in ice

for 20 minutes. The tubes were centrifuged at 13,000rpm for 15 minutes and the supernatant was discarded. The pellet (precipitated protein) was washed with 500µl wash solution. (Vortex well to make sure the solution reaches even the inside of the cap of the tubes). Centrifuge at 13,000rpm for 15 minutes and pellet the protein. This step was repeated two more times. After third wash, short spin the tubes for 30 secs to 1 min at high speed and all remaining wash solution was removed. Air-dry the protein samples in both tubes for a few minutes- until there was no visible liquid in the tube. The protein was dissolved in 400µl of guanidine HCl solution. Generally, TCA precipitated protein will take time to dissolve. The tube was vortex for 30 seconds. 200µl of the sample was transferred from both tubes into wells in 96 well-plate and the absorbance was read at 370nm. The results were calculated by applying Beer-Lambert's Law (explained below) and is expressed in the unit nmol carbonyl/mg protein.

$$Concentration\ (Initial) = \left(\frac{Adjusted\ Absorbance}{22\ \times 0.5349}\right) \times 200$$

Where,

$Adjusted\ Absorbance = Test\ Absorbance - Control\ Absorbance$

22mM-1cm-1 is the molar extinction co-efficient of aliphatic hydrazones 0.5349cm is the path length for 200µl in Hellma 96 well quartz plate which is calculated using the formula,

$$Pathelength = \frac{(4 \times Volume\ in\ well)}{(\prod \times Diameter^2)}$$

Concentration (Initial) corresponds to the concentration in nmol of protein carbonyl in 200µl of the sample in microplate. In order to get the final concentration in nmol/mg protein, the protein concentration value has to be substituted to the value.

Therefore,

$$Protein\ concentration\ in\ well = \frac{\left(\frac{Protein\ concentration\ of\ sample\ in\ mg/ml}{10}\right)}{2}$$

$$Final\ concentration\ in\ \frac{nmol\ carbonyl}{mg\ protein} = \frac{Concentration\ Initial}{Protein\ concentration\ in\ well}$$

3.5 Inflammatory Markers

Immunoassays & MILLIPLEX® map Kits was used to measure IL-6, TNF-alpha, IFN_Y and VEGF concentrations in organ [97]. Assay started with the quality control preparation, before use, Quality Control 1 and Quality Control 2 were reconstituted with 250 µL deionized water. The vial was inverted several times to mix and vortex. Then allowed to sit for 5-10 minutes. Unused portion was stored at -20°C for up to one month. Then, the 10X Wash Buffer was brought to room temperature and mixed to bring all salts into solution. Then 60 mL of 10X Wash Buffer (two bottles) was diluted with 540 mL deionized water. Rat Cytokine / Chemokine Standard was reconstituted with 250 µL deionized water. The vial was inverted several times to mix. The vial was vortex for 10 seconds and then allowed to sit for 5-10 minutes. This was used as Standard 7.

The Working Standards was prepared by first labeling 6 polypropylene microfuge tubes Standard 1 through Standard 6, then add 120 µL of Assay Buffer to each of the 6 tubes. The serial dilutions were prepared by adding 40 µL of the reconstituted Standard 7 to the Standard 6 tube, mixed well and transferred 40 µL of Standard 6 to the Standard 5 tube, mixed well and transferred 40 µL of Standard 5 to

the Standard 4 tube, mixed well and transferred 40 µL of Standard 4 to the Standard 3 tube, mixed well and transferred 40 µL of Standard 3 to the Standard 2 tube, mixed well and transferred 40 µL of Standard 2 to the Standard 1 tube and mixed well. The 0 ng/mL standard (Background) was the Assay Buffer. The immunoassay procedure started by allowing all reagents to warm to room temperature (20-25°C) before use in the assay. 200 µL of Assay Buffer was added into each well of the plate. Sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25°C).

Assay Buffer was decanting and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. 25 µL of each Standard or Control was added into the appropriate wells. Assay Buffer was used for 0 ng/mL standard (Background). 25 µL of Assay Buffer was added to the sample wells and 25 µL of appropriate matrix solution was added to the background, standards, and control wells. 25 µL of Sample (1:2 diluted) was added into the appropriate wells. Then the Mixing Bottle was vortex and 25 µL of the Mixed or Premixed Beads was added to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.), after that the plate was sealed with a plate sealer. The plate was wrapped with foil and incubated with agitation on a plate shaker 2 hours at room temperature (20- 25°C). The contents were gently removed well and the plate was washed 2 times following instructions listed in the PLATE WASHING section and 25 µL of Detection Antibodies was added into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.).

The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). 25 µL Streptavidin-

Phycoerythrin was added to each well containing the 25 µL of Detection Antibodies. The plate was Sealed, covered with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C). The contents were gently remove well and the plate was washed 2 times following instructions listed in the PLATE WASHING section. 125 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) was added to all wells. Resuspend the beads on a plate shaker for 5 minutes. The plate was run on Luminex 200TM, HTS, FLEXMAP 3DTM or MAGPIX® with xPONENT software. Rat cytokine / Chemokine magnetic bead panel kit 2012 [97].

3.6 Histopathology

Following different organ procurement, tissue fragments were placed in 10% buffered formalin for 8 hours. Manual tissue processing involved dehydration by using ethyl alcohol, clearing by using xylene and infiltration by paraffin. Four-micrometer sections were obtained from paraffin blocks and stained with hematoxylin and eosin. Fragments of liver, muscle, heart and brain were prepared.

3.7 Statistical Analysis

Statistical analysis performed by using SPSS windows (Version 23; SPSS Inc. Chicago, Illinois, USA). Means ± standard error (s.e.) were calculated for each group, each parameter, in serum and in each organ. Post-hoc Tukey tests were used to compare the results between DSP1, DSP2, DSP3 and control groups in serum and in each organ. Statistical significance was set at $P < 0.05$.

Chapter 4: Results

4.1 Biochemical Results

Biochemical parameters in the different groups are shown in Table 1. Glucose, cholesterol, HDL-C, total serum protein, and albumin were not significantly different between groups. In contrast, LDH and CK, which are biomarkers of high levels of tissue damage, were higher in controls compared to the DSP groups. Urea and creatinine are indicators of kidney function. They were reduced with DSP compared to the control, but the results were not significant, except for urea, which was significantly lower in DSP3 compared to DSP1. Some differences were observed for liver enzymes. The consumption of DSP was associated with a significant decrease in the levels of both AST and GGT compared to the control group in a dose-dependent manner. A similar trend was observed for ALT, which was significant only in DSP1 and DSP3 compared to the control.

Table 1: Biochemical parameters in the control and date seed powder groups

	Control	DSP1	DSP2	DSP3
Blood glucose (m mol/L)	7.74 ± 0.79	7.82 ± 0.59	9.62 ± 0.64	9.58 ±0.42
Blood cholesterol (mmol/L)	1.78 ± 0.05	1.60 ± 0.11	1.61 ± 0.12	1.53 ± 0.11
Blood HDL-C (mmol/L)	1.09 ± 0.06	1.11 ± 0.69	1.06 ± 0.08	0.96 ± 0.06
Total blood protein (g/L)	66.60 ± 1.92	64.38 ± 1.19	65.37 ± 1.16	64.30 ± 0.82
Blood albumin (g/L)	41.38 ± 0.99	40.53 ± 0.35	40.83 ± 0.63	41.63 ± 0.60
Blood LDH (IU/L)	3690.60 ± 212.57	2647.50 ± 249.47[a]	2507.37 ± 202.83[a]	2074.0 ± 179.27[a]
CK (IU/L)	11701.25 ± 846.36	3881.17 ± 1367.47[a]	2103.20 ± 345.90[a]	3875.90 ± 467.51[a]
Urea (mmol/L)	4.96 ± 0.24	5.25 ± 0.23	5.09 ± 0.25	4.23 ± 0.25[b]
Creatinine (mmol/L)	35.58 ± 1.05	32.06 ± 2.82	30.76 ± 3.03	26.31 ± 1.56
ALT (IU/L)	62.60 ±10.67	36.30 ± 4.46 [a]	37.00 ± 6.65	37.00 ± 4.77 [a]
ALP (IU/L)	97.05 ± 7.21	89.37 ± 5.60	89.64 ± 12.13	95.14 ± 5.85
AST (IU/L)	337.60 ± 41.38	201.00 ± 40.16[a]	170.75 ± 20.95[a]	149.60 ± 9.24[a]
GGT (IU/L)	5.75 ± 1.25	1.50 ± 0.22[a]	1.25 ± 0.16[a]	1.20 ± 0.13[a]

Means ± s.e. are presented[*].

[*]Means were compared between groups using post-hoc Tukey's test. Statistical significance was set at $P < 0.05$.
[a]: Statistically significant difference with the control group
[b]: Statistically significant difference with the DSP1 group
[c]: Statistically significant difference with the DSP2 group

4.2 Antioxidant Status Biomarkers

The blood levels of different biomarkers of the oxidative status are presented in Table 2. Vitamin C and vitamin E were both increased with DSP compared to the control, but it was not significant. ABTS was significantly reduced in DSP2 and DSP3 compared to the control.

Table 2: Biomarkers of the antioxidant status in the blood of the control and date seed powder groups

	Control	DSP1	DSP2	DSP3
Vitamin C (μg/ml)	0.05 ± 0.01	0.04 ± 0.00	0.05 ±0.01	0.08 ± 0.04
Vitamin E (μg/ml)	2.01 ± 0.32	2.74 ± 0.20	2.64 ± 0.34	3.00 ± 0.37
ABTS %	75.93 ± 0.75	73.25 ± 0.48	72.86 ± 0.76[a]	73.03 ± 0.61[a]

Means ± s.e. are presented.[*]

[*]Means were compared between groups using post-hoc Tukey's test. Statistical significance was set at $P < 0.05$.
[a]: Statistically significant difference with the control group
[b]: Statistically significant difference with the DSP1 group
[c]: Statistically significant difference with the DSP2 group

4.3 Biomarkers of Oxidative Status in Organs

Table 3 shows the levels of different biomarkers of oxidative status in the liver, muscle, heart and brain. In the liver, both vitamin C and vitamin E were increased with DSP, but it was not significant. In contrast, GSH was significantly increased with DSP, especially in DSP3, compared to the control. In muscle, both vitamin C and vitamin E tended to increase compared to the control, but it was not significant. GSH increased with DSP and the increase was significantly different from the control with all three doses of DSP. In the heart, a significant difference was only observed for GSH, which was higher with DSP. In the brain, all the biomarkers measured did not change with DSP.

Table 3: Biomarkers of the antioxidant status in the blood of the control and date seed powder groups

	Control	DSP1	DSP2	DSP3
Liver				
Vitamin C (mcg/g)	0.30 ± 0.026	0.25 ± 0.06	0.49 ± 0.10	0.65 ± 0.16
Vitamin E (mcg/g)	23.94 ± 2.67	41.36 ± 8.99	49.06 ± 4.80	36.01 ± 3.72
GSH (nmol/g)	568.80 ± 5.71	589.89 ± 13.60	572.91 ± 9.78	631.46 ± 16.18 [a, c]
Muscle				
Vitamin C (mcg/g)	8.74 ± 1.02	10.91 ± 1.63	10.08 ± 0.36	9.31 ± 0.76
Vitamin E (mcg/g)	5.52 ± 0.73	4.72 ± 0.71	6.67 ± 0.40	6.50 ± 0.37
GSH (nmol/g)	159.91 ± 18.14	258.86 ± 17.15[a]	293.25 ± 16.23[a]	312.02 ± 8.70 [a, b]
Heart				
Vitamin C (mcg/g)	26.20 ± 1.34	21.02 ± 2.53	24.07 ± 1.22	20.54 ± 0.95
Vitamin E (mcg/g)	73.13 ± 0.08	64.94 ± 8.12	72.92 ± 0.03	72.96 ± 0.02
GSH (nmol/g)	475.98 ± 12.04	505.46 ± 10.94	490.14 ± 10.33	668.83 ± 36.77 [a,b,c]
Brain				
Vitamin C (mcg/g)	63.50 ± 3.06	59.28 ± 6.70	69.50 ± 3.27	66.91 ± 2.50
GSH (nmol/g)	487.16 ± 20.38	469.25 ± 5.94	480.33 ± 9.01	476.48 ± 10.56

Means ± s.e. are presented.[*]

[*]Means were compared between groups using post-hoc Tukey's test.
Statistical significance was set at $P < 0.05$.
[a]: Statistically significant difference with the control group
[b]: Statistically significant difference with the DSP1 group
[c]: Statistically significant difference with the DSP2 group

4.4 Oxidative Damage Biomarkers

The level of protein-bound carbonyls, biomarker of protein oxidative damage, in serum and organs, respectively, are shown in Figure 1 & Figure 2. Protein-bound carbonyls were reduced only in muscle and significantly only in DSP1 compared with control. In serum and other organs, it tended to decrease with DSP without statistical significance.

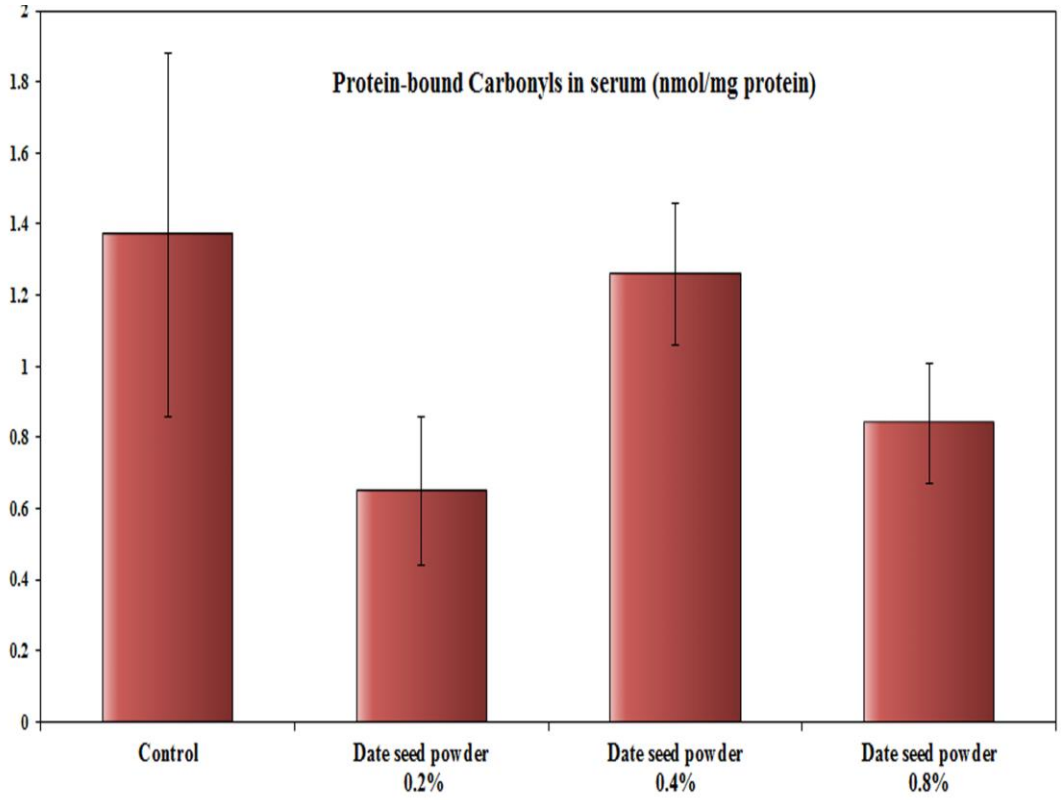

Figure 8: Protein-bound Carbonyl in Serum (nmol/mg protein)

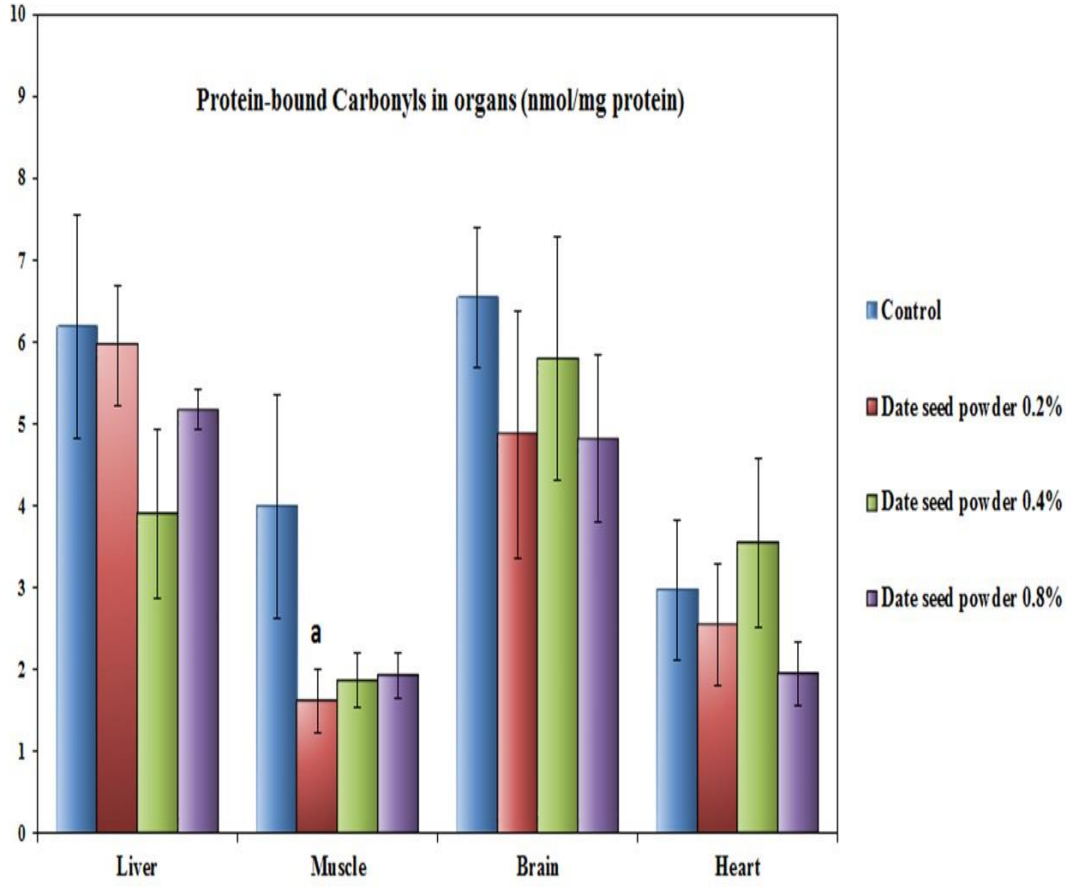

Figure 9: Protein-bound Carbonyls in Organs (nmol/mg protein)
Means ± s.e. are presented*

* Means were compared between groups using post-hoc Tukey's test. Statistical significance was set at $P < 0.05$.
a: Statistically significant difference with the control group
b: Statistically significant difference with the DSP1 group
c: Statistically significant difference with the DSP2 group

The level of MDA, biomarkers of lipid oxidative damage, is serum and organs, respectively, are shown in Figure 3 & Figure 4. MDA was significantly reduced in the liver, muscle and brain. In the liver, MDA was decreased with DSP1 and DSP3, while in the brain and muscle, it was significantly reduced in all treated groups in a dose-dependent manner compared to controls. In serum, it tended to decrease with DSP without statistical significance.

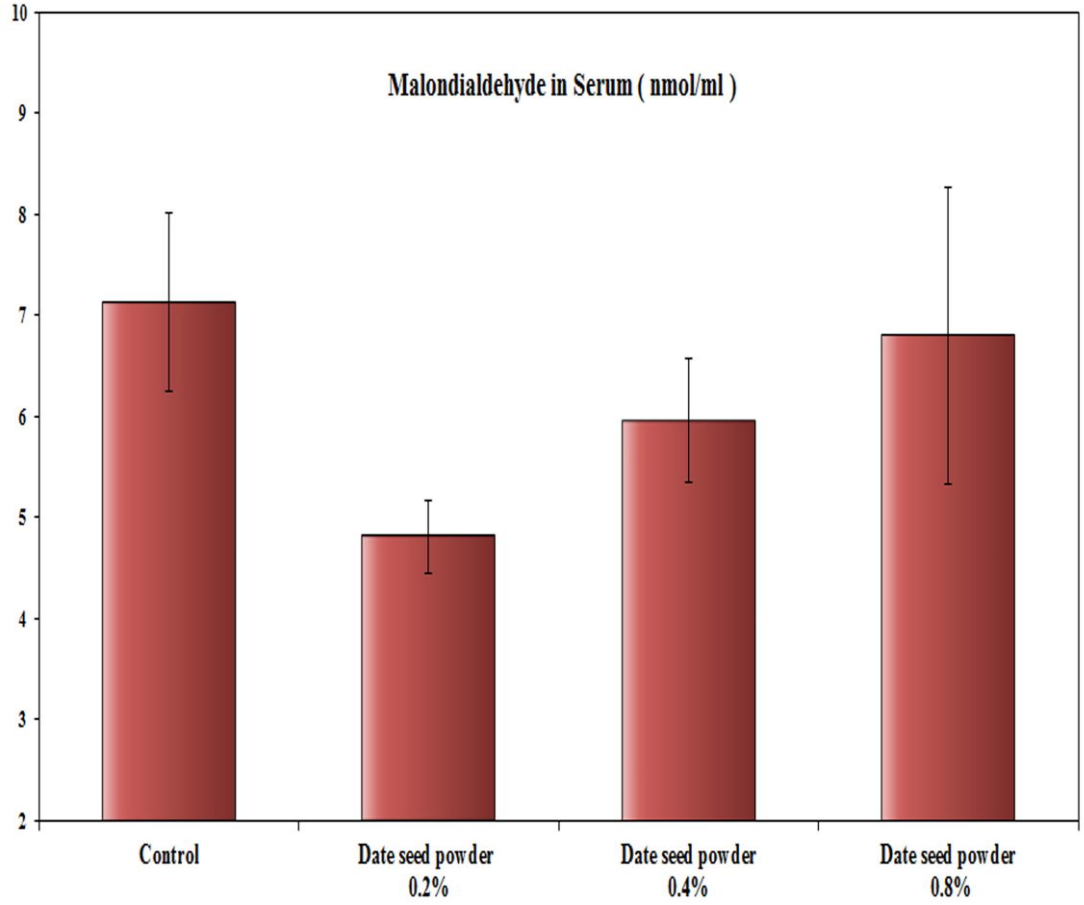

Figure 10: Malondialdehyde in Serum (nmol/ml)

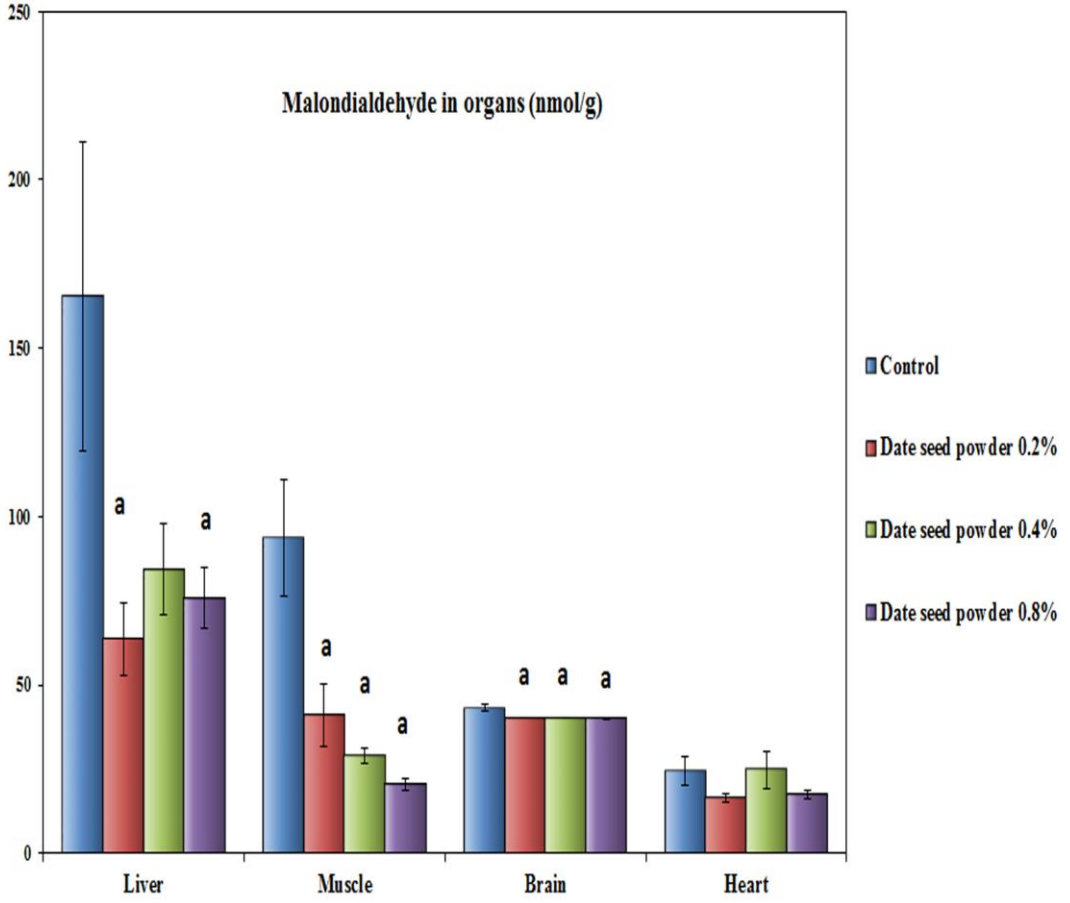

Figure 11: Malondialdehyde in Organs (nmol/ g)
Means ± s.e. are presented*

* Means were compared between groups using post-hoc Tukey's test. Statistical significance was set at $P < 0.05$.
a: Statistically significant difference with the control group
b: Statistically significant difference with the DSP1 group
c: Statistically significant difference with the DSP2 group

4.5 Markers of Low-grade Inflammation in the Brain and Liver

Table 3 shows the levels of inflammatory markers, IL6, IFNγ and VEGF, in the brain. For IL-6 and VEGF biomarkers, their levels tended to increase with DSP, especially with DSP2, compared to the control, then to decrease again in DSP3 group. But none of the differences was statistically significant. While for IFNγ there was a trend of decrease especially with DSP1 and DSP3 but also the decrease was not significant.

Table 4: Markers of the Brain inflammation in control and date seeds powder groups

	Control	DSP1	DSP2	DSP3
IL-6 (pg/ml)	3.53 ± 0.60	4.52 ± 0.42	4.92 ± 0.26	4.69 ± 0.21
IFNγ (pg/ml)	1.72 ± 0.25	1.50 ± 0.26	1.94 ± 0.37	1.05 ± 0.13
VEGF (pg/ml)	2.34 ± 0.53	3.25 ± 0.37	3.80 ± 0.43	2.91 ± 0.24

Table 4 shows the levels of inflammation markers (IL-6, IFNγ, VEGF and TNFα) in the liver. No difference between the groups was statistically different. However, there was a trend of increase in IL-6 and VEGF markers with DSP.

Table 5: Markers of the Liver inflammation in control and date seeds powder groups

	Control	**DSP1**	**DSP2**	**DSP3**
IL6 (pg/mg)	31.66 ± 6.0	38.24 ± 1.85	36.07 ± 1.84	39.23 ± 2.23
IFNγ (pg/mg)	17.02 ± 1.72	18.98 ± 1.28	17.50 ± 0.82	17.96 ± 0.68
VEGF (pg/mg)	13.00 ± 1.66	12.64 ± 1.12	15.31 ± 1.48	13.98 ± 0.83
TNFα (pg/mg)	0.36 ± 0.06	0.43 ± 0.03	0.43 ± 0.04	0.45 ± 0.04

4.6 Histopathology

Liver, muscle, heart and brain samples of control rats and rats from DSP1, DSP2 and DSP3 all showed a normal appearance (Figure 2). No changes related to ischemia were detected. These observations confirm the absence of any changes related to the oral consumption of DSP by rats.

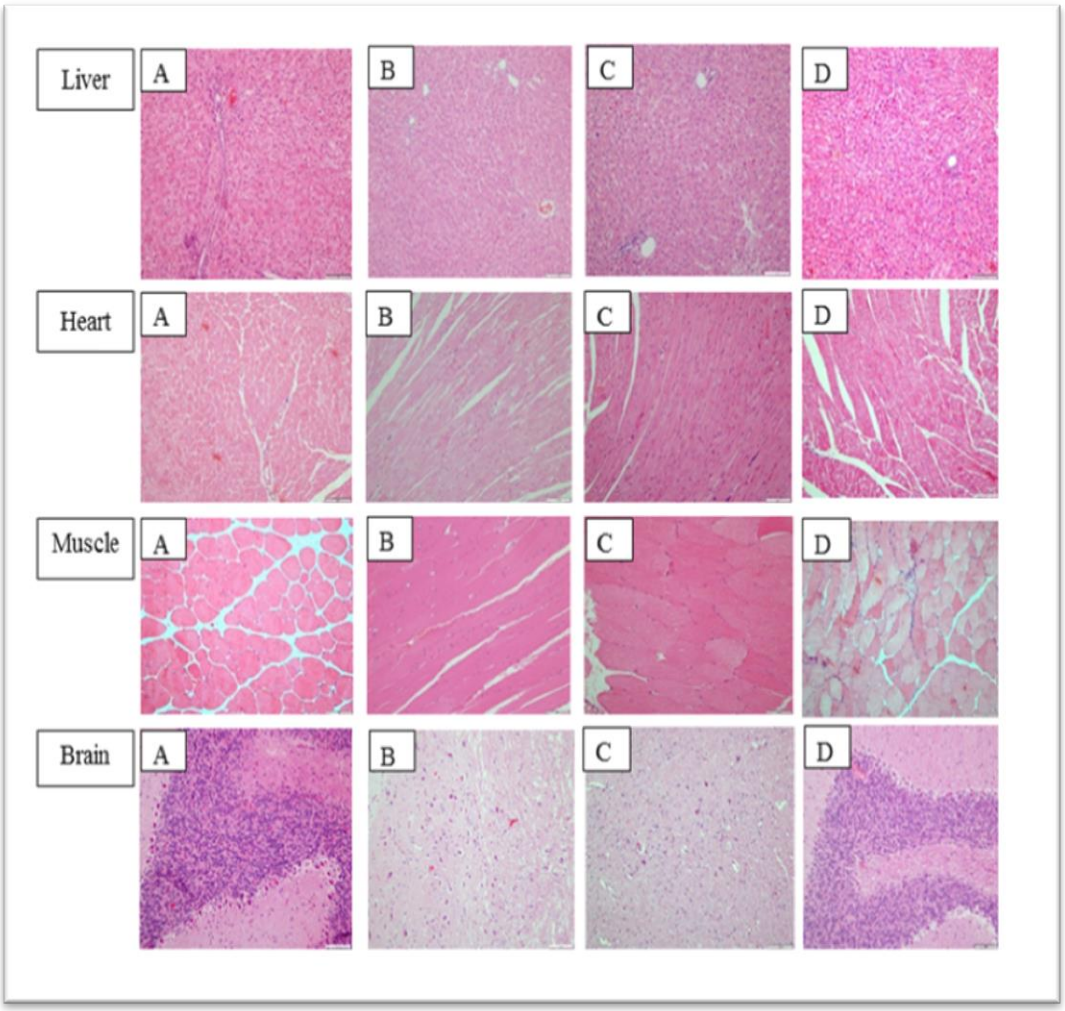

Figure 12: Histopathological Observations in Liver, Muscle, Heart and Brain Hematoxylin and eosin ×20. (A) Control group, (B) date seed powder 0.2%, (C) date seed powder 0.4%, (D) date seed powder 0.8%.

Chapter 5: Discussion

The main purpose of this study was to investigate the safety aspects and effects of DSP on antioxidant status and oxidative damage in animals. The results clearly show that DSP did not display any significant toxic effect and did not alter organ function. The main biochemical indicators were not altered by the consumption of DSP and no tissue damage was discovered. In addition, DSP was shown to strengthen the defenses of the antioxidant system and protect against oxidative damage.

5.1 The Safety of DSP in Animals

Consumption of DSP was shown to be safe in rats due to no changes occurring in the main biochemical parameters even after consumption of DSP. Urea and creatinine, both nitrogenous end products of metabolism that are used in screening renal function, did not change significantly under the influence of DSP. As elevated levels of urea and creatinine indicate renal disease, this indicates that DSP is not toxic and does not interfere with renal function. Additionally, intracellular enzymes, including ALT, AST, ALP, GGT and LDH, all indicators of tissue injury, especially in the liver where ALT, AST and ALP are concerned were considered [98] [99]. An increase in these parameters has been related to hepatic, pancreatic and skeletal muscle diseases [100]. Their decrease due to DSP indicates that DSP did not alter tissue function and conversely can improve this function through protecting these organs. A similar decrease has been observed in a previous study exploring DSP [16].

This could be due to the polyphenol content of DSP, especially the phenolic compounds. Indeed, these compounds were found to reduce the leakage of liver enzymes into the blood by providing protection against free radicals that may cause tissue damage [101; 16; 102].

5.2 DSP and Oxidative Balance

Interestingly, DSP has been associated with the increased defense of the antioxidant system in the serum and organs. This is shown by a reduction of ABTS in the serum and increased GSH in the organs. The ABTS test assesses the antioxidant effect of natural compounds found in food or other biological systems. It generates when a strong oxidizing agent, such as potassium persulfate, reacts with the ABTS salt producing a blue-green color. The ability of natural compounds to reduce the dark blue-green-colored ABTS free radicals to a colorless form provides evidence for the strength of the antioxidant [103].

Glutathione is a small tripeptide synthesized from amino acids in the liver. It is a vital intracellular and extracellular protective antioxidant and is involved in many cellular functions such as control of the signaling process. It also serves as a co-factor in many detoxifying enzymes related to oxidative stress and xenobiotics. It can also help in the regeneration of important antioxidant vitamins, such as vitamins C and E. The depletion of GSH characterizes the aging process and many degenerative and apoptotic conditions. Consequently, an increase of GSH strengthens the antioxidant system by enhancing its protective effect against free radicals [104]. An elevation in such levels was observed in our study on DSP in the liver, muscle and heart. This increase in GSH could be related to a high level of date seed polyphenols increasing the expression of γ-glutamylcysteine synthetase, which

is an enzyme that limits the synthesis of GSH [105]. In addition, this might be explained by the fact that date seeds are rich in catechins, which have been shown in several studies to regulate-up anti-oxidant enzymes, including GSH. Moreover, catechins have been observed to increase plasma and tissue glutathione levels in several studies on animals [13]. Surprisingly, there was no increase of GSH in the brain, even though GSH is part of many central nervous system activities as both a neuromodulator and a neurotransmitter. It is also a major antioxidant in the brain. The production of free radicals is high in brain tissue due to high utilization of oxygen. However, GSH levels in the brain are low compared to other organs. This could be due to a tight regulation of GSH levels thanks to the toxicity of the substrates used to synthesize GSH in the neurons [106].

Vitamin C (ascorbic acid) is a water-soluble molecule that reacts directly with the free radicals. Additionally, it can regenerate a reduced antioxidant form of Vitamin E. Vitamin E (tocopherol) is a lipid-soluble antioxidant found in every cell membrane. It protects the cells from attack by free radicals and prevents lipid peroxidation [107]. Although it did not reach a significant level, both vitamins tended to increase along with an increase in DSP. In fact, Habib and Ibrahim (2011) reported a similar result for Vitamin E [16].

As far as Vitamin C is concerned, this lack of significance may be explained by the fact that rats, unlike humans, have the ability to synthesize ascorbic acid in their livers due to hepatic L-gulonolactone oxidase. Additionally, rats exhibit poor ascorbic acid absorption, meaning that ascorbic acid's function in maintaining an antioxidant function is less important in rats than the other important functions carried out by this nutrient [108]. With vitamin E, the wide variability noted and the

sensitivity of the method utilized cannot be ignored. Additionally, Vitamin E can only be obtained through diet. After ingestion, a rapid increase in plasma and in the main storage organs, such as the muscles, is exhibited. This indicates the direct regulation of the exchange between plasma and metabolically active organs. In addition, fast recycling mechanisms that maintain Vitamin E in the blood in a reduced state exist [109; 110]. Furthermore, the trend observed for Vitamin E levels can also be related to greater levels of GSH. Indeed, GSH interacts with Vitamin E to maintain it in its reduced form [111]. This can be attributed to date seed catechins. Previous studies have shown that catechins participate in Vitamin E recycling, and thus complement the function of glutathione [13].

Finally, the protective effect of DSP against protein and lipid oxidative damage has been observed. Generation of ROS can cause damage to many molecules in the body, including proteins. One of the most widely used indicators of the products of protein oxidation a protein-bound carbonyl. It is generated from protein oxidation either through a glutamyl side chain oxidation or an α-amidation pathway. This oxidation process results in the formation of a peptide with an N-terminal amino acid, which is, in turn, blocked by α-ketoacyl derivatives. The accumulation of protein-bound carbonyls has been observed in many diseases, including diabetes, Alzheimer's disease and arthritis [112]. In this study, protein-bound carbonyls tended to decrease in relation to DSP in every group, thus supporting the idea of DSP having a protective effect against oxidative protein damage. Nevertheless, the reduction was significant only in muscles with DSP at a concentration of 0.2%. This can be explained by the fact that skeletal muscles represent approximately 40% of mammalian body weight, and at least 25% of the protein turnover occurs in these tissues. In addition, skeletal muscles include many other proteins that are involved in

performing muscle functions and produce high levels of ROS due to their high metabolic rate [113]. Therefore, the protective effect of DSP against oxidative protein damage is expressed more intensely in these muscles.

In terms of indicators of lipid peroxidation, this study used MDA. This choice was made, as it is the most extensively studied product of lipid peroxidation. MDA is one of the major aldehydes that result from the breakdown of lipid hydroperoxides. It is considered as a good biomarker of pathologic damage as it is linked to oxidative stress [114].

MDA was significantly reduced across every DSP concentration in the liver, muscle and brain. Meanwhile in the serum and heart, the reduction was not significant. Habib, et al. [16], who studied livers 30 days after consumption, obtained similar results. Serum and heart MDA levels can be affected by a blood sampling tube, the stability of the biomarker under different storage conditions, the sensitivity and ability to reproduce the method and also the steps involved in specimen preparation. In addition, the serum's antioxidant levels may be affected by differing factors such as homeostatic regulation and the degree of absorption [28; 115; 116; 117].

Other studies were not able to illustrate a direct antioxidant effect of the biological application of polyphenols on cardiovascular health. Some foods rich in polyphenols have positive effects on some biomarkers of cardiovascular health. However, there is no concrete evidence that an improvement in antioxidant biomarkers and markers of oxidative damage, such as MDA, represent a true benefit to health [118].

The results above strongly support the potential effect of DSP in protecting against oxidative stress and its related organ damage *in vivo*. This is mainly through its antioxidant effect and by strengthening the endogenous antioxidant system.

5.3 DSP and the Prevention of Inflammation

Another purpose of this study was to investigate the potential anti-inflammatory effects of DSP in rats by evaluating low-grade inflammation cytokines such as IL-6, TNFα, IFN-γ and VEGF in the liver and brain.

The role of DSP in decreasing markers of inflammation has not been fully investigated. This is the first study to properly investigate the anti-inflammatory properties of DSP. Our results did not show a significant effect of DSP intake on liver and brain levels in terms of IL-6, TNF-a, IFN-γ and VEGF. It should be noted that the inflammatory markers in our samples were in the normal physiologic concentration range at the outset of the study. DSP might affect these biomarkers by creating a higher initial pro-inflammatory status that suggest a therapeutic rather than preventive role for DSP in terms of inflammation.

Low-grade inflammation was defined as a condition where pro-cytokines such as TNFα increase 2 to 3-fold. A lot of evidence highlights the role of TNFα in low-grade inflammation, in the activation of other pro-inflammatory cytokines, and its direct role in glucose and lipid metabolism impairment [119; 120], as well as in the development of metabolic syndrome. For instance, an increased level of TNFα has been found to increase the synthesis and accumulation of triglycerides in the liver, by inhibiting peripheral tissue lipolysis [120].

Our results clearly showed that there was no significant change in the TNFα levels between the control group and the DSP group in a rat liver. The TNFα level was maintained within minimum standard values. This indicates an absence of low-grade inflammation.

This might be related to an increase in Il-6, especially in the liver. IL-6 is one of the most important members of the cytokine family, which has a broad effect on many cells types, whether related to the immune system or not. IL-6 is an acute phase reactant cytokine that has context-dependent pro- and anti-inflammatory properties with pleiotropic biological effects. Additionally, it displays a hormone-effect that contributes to homeostatic processes [121].

The liver is an important source of IL-6 and the primary site for clearing it out. IL-6 is a critical cytokine required to control and modulate other cytokines such as TNFα and IFN-γ. It induces the release of anti-inflammatory molecules such as IL-1 and a TNFα receptor antagonist [122]. It also releases the soluble receptor p55, a molecule that competes with the membrane bound receptors for the circulation of TNFα [123]. The potential uses of this mechanism are supported by studies which demonstrate that polyphenols, such as catechins and epicatechins, can inhibit the expression and secretion of pro-inflammatory molecules, like TNFα, whilst enhancing the production of anti-inflammatory cytokines such as IL-6 [6]. Therefore, the increase in Il-6 demonstrated in our study can be attributed to DSP polyphenol consumption, especially DSP2. This could have resulted from the suppression of TNFα.

Recent studies indicate IL-6 induces hepatocyte proliferation through the translocation of cytoplasmic STAT 3 (a signal transducer and activator of

transcription protein 3) into the nucleus. This binds to DNA and induces several promoters that lead to a hepatoprotective effect [123]. This hepatoprotective effect was observed in our study and has been described above.

Another possible mechanism for down regulation of TNFα is the ability of polyphenols to affect immune functions by modulating the inflammatory response in macrophage cells. These cells are known to play a major role in the initiation of inflammatory responses by producing pro-inflammatory cytokines, such as TNFα [124]. There is evidence that fruit and tea flavonoids, especially epicatechin, one of the main polyphenol classes in DSP, have immune-modulatory properties. These operate in terms of chemokine inhibition in different cells and regulate-down TNFα receptor 1 [125].

Another important inflammatory agent tested in this study is VEGF. VEGF signaling is required for normal vascular development, homeostasis and as an essential growth factor in vascular endothelial cells [126]. However, VEGF is a potent factor in increasing endothelial cell permeability that, in turn, leads to plasma components and leukocytes passing from blood vessel into tissues and contributing to inflammatory responses. Furthermore, VEGF is the most important factor in the angiogenesis process whereby new blood vessels are formed from pre-existing ones. An abundant VEGF expression has been observed in human atherosclerotic plaques [6]. Contrary to other studies, which have highlighted the inhibitory effect of epicatechins on VEGF expression, our study found no significant change in the VEGF level. It maintained minimum standard values with every DSP treatment [127].

In terms of IFN-γ levels, our results showed that there was no effect from DSP on the different groups. Thus, DSP did not cause inflammation in rat tissue. This enhances its potential for safe consumption considerably. However, there was a decrease in the IFN-γ level in brain tissue, especially with DSP1 and DSP3, when compared to the control group. That said it was not a significant difference. IFN-γ is recognized as a chief mediator cytokine with innate as well as adaptive immunity. It is responsible for various key biological activities including the activation of macrophages. IFN-γ may also be able to enhance the pro-inflammatory transcription factor known as nuclear factor-κB (NF-κB) under certain conditions [128].

In certain previous studies, polyphenols showed immune-modulatory effects to protect against inflammation. For example, this included reducing the expression of a wide range of neuro-inflammatory markers such as IFN-γ [129]. Due to its capacity to modulate important inflammatory markers, DSP holds out great promise in terms of potentially therapeutic strategies to control inflammation and related diseases. Thus, our DSP treatment may also display immune-modulatory effects due to its polyphenol content.

5.4 Limitations

Some limitations of the study should be noted. Firstly, polyphenol absorption and the metabolism are complex processes that still require deeper investigation, especially in terms of issues related to polyphenol dosage and bioavailability. In addition, most of in vivo polyphenol studies have been conducted on a short-term basis and therefore more longitudinal studies are required in order to determine the long-term effects of these diverse compounds [6].

Moreover, the lack of significance in some of our findings was probably affected by technical limitations and the characteristics of the study such as the duration of the treatment. The possibility of protein degradation due to -80^0 C storage of the biological samples cannot be excluded. Although most cytokines are stable for up to 2 years of storage at −80°C, other cytokines such as IL-6 are degraded by up to 50% within 2–3 years of storage. Additionally, the levels of circulating cytokines can be affected by sample collection protocols such as handling, processing, storage and even animal behaviour prior to collection. Another important limitation is the short half-life of cytokines. For example, the half-life of IL-6 is <6 hours. Even though our samples have been analysed using multiplex arrays, which is more suitable than ELISA, the use of multiplex arrays in clinical research remains limited. Multiplex results can be influenced by the abundance of proteins circulating, as bead-based multiplex array reactions take place in molecules and antigens, which are freely mobile in solution. As a result, multiplex arrays appear to be much more sensitive than ELISAs are to altered levels of circulating proteins and inhibitors. As many circulating proteins, may change their levels during aging, inflammation or disease, this can further complicate the picture. Therefore such new technologies need to be used with caution [130].

Chapter 6: Conclusion

The results of this study clearly show that DSP was safe and did not alter any tissue functions. This was shown by conducting an analysis of the biochemical parameters, histopathology and immune assay results. In addition, DSP exhibited a protective effect against oxidative stress *in vivo*, mainly due to its antioxidant potential and by strengthening the endogenous antioxidant system. Thus, DSP is a potential candidate for reducing the markers of oxidative damage, especially MDA, in bodily organs. DSP also did not alter the immune inflammation markers. This gives support to the safe consumption of DSP as well as suggesting its possible anti-inflammatory effects.

Therefore, if we consider the role of oxidative stress as a mechanism underlying chronic disease, it can be concluded that DSP can contribute to the prevention of these diseases. Although polyphenols are at least partly responsible for the effects of DSP *in vivo*, the underlying mechanisms require further investigation. Future experimental design should consider the true absorption and bioavailability of polyphenols, as well as the fact that during polyphenol digestion and absorption, they may undergo a considerable degree of chemical modification that can alter their biological properties and potency. Obviously, further studies are required to understand the effect of ROS on basic cellular functions. We also need to better understand how different cells respond to the ROS effect and how this, in turn, influences the pathology of different inflammatory diseases.

References

1. Organization WH. Country profiles. Noncommunicable diseases country profiles 2014.

2. Organization WH. The world health report 2003: shaping the future: World Health Organization; 2003.

3. Diabetes Facts 2011 [cited 2016 04 January]. Available from: http://diabetesuae.ae/diabetes/diabetes-facts/.

4. WHO J, Consultation FE. Diet, nutrition and the prevention of chronic diseases. World health organ tech rep ser 2003; 916.

5. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. The American journal of clinical nutrition 2003; 78:517S-20S.

6. Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. Biochemical pharmacology 2006; 72:1439-52.

7. Rodrigo R, Libuy M, Feliú F, Hasson D. Oxidative stress-related biomarkers in essential hypertension and ischemia-reperfusion myocardial damage. Disease markers 2013; 35:773-90.

8. Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. The American journal of clinical nutrition 2005; 81:215S-17S.

9. Habib HM, Ibrahim WH. Nutritional quality of 18 date fruit varieties. International journal of food sciences and nutrition 2011;62:544-51.

10. Al-Shahib W, Marshall RJ. The fruit of the date palm: its possible use as the best food for the future? International journal of food sciences and nutrition 2003; 54:247-59.

11. Platat C, Habib HM, Maqbali FDA, Jaber NN, Ibrahim WH. Identification of date seeds varieties patterns to optimize nutritional benefits of date seeds. Journal of nutrition & food sciences 2014; 2014.

12. Habib HM, Platat C, Meudec E, Cheynier V, Ibrahim WH. Polyphenolic compounds in date fruit seed (Phoenix dactylifera): characterisation and quantification by using UPLC-DAD-ESI-MS. Journal of the science of food and agriculture 2014;94:1084-89.

13. Velayutham P, Babu A, Liu D. Green Tea Catechins and Cardiovascular Health: An Update. Current medicinal chemistry 2008;15:1840-50.

14. Jeong W-S, Kong A-NT. Biological properties of monomeric and polymeric catechins: Green tea catechins and procyanidins. Pharmaceutical Biology 2004;42:84-93.

15. Simos YV, Verginadis II, Toliopoulos IK, Velalopoulou AP, Karagounis IV, Karkabounas SC, Evangelou AM. Effects of catechin and epicatechin on superoxide dismutase and glutathione peroxidase activity, in vivo. Redox report 2012;17:181-86.

16. Habib HM, Ibrahim WH. Effect of date seeds on oxidative damage and antioxidant status in vivo. Journal of the science of food and agriculture 2011;91:1674-79.

17. Al-Farsi M, Lee C. Usage of date (Phoenix Dactylifera L.) seeds in human health and animal feed. Nuts and seeds in health and disease prevention p 2011:447-52.

18. Ali B, Bashir A, Alhadrami G. Reproductive hormonal status of rats treated with date pits. Food chemistry 1999;66:437-41.

19. Al-Qarawi AA, Mousa HM, Ali BH, Abdel-Rahman H, El-Mougy SA. Protective effect of extracts from dates (Phoenix dactylifera L.) on carbon tetrachloride-induced hepatotoxicity in rats. Int j appl res vet med 2004;2:176-80.

20. Platat C, Habib HM, Hashim IB, Kamal H, AlMaqbali F, Souka U, Ibrahim WH. Production of functional pita bread using date seed powder. Journal of food science and technology 2015;52:6375-84.

21. Sies H. Oxidative stress: oxidants and antioxidants. Experimental physiology 1997;82:291-95.

22. Devasagayam T, Tilak J, Boloor K, Sane KS, Ghaskadbi SS, Lele R. Free radicals and antioxidants in human health: current status and future prospects. Japi 2004;52:4.

23. Guetens G, Boeck GD, Highley M, van Oosterom AT, de Bruijn EA. Oxidative DNA damage: biological significance and methods of analysis. Critical reviews in clinical laboratory sciences 2002;39:331-457.

24. Mayne ST. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. The Journal of nutrition 2003;133:933S-40S.

25. Uttara B, Singh AV, Zamboni P, Mahajan R. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Current neuropharmacology 2009;7:65-74.

26. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity 2009;2:270-78.

27. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. Journal of clinical investigation 2004;114:1752-61.

28. Ho E, Galougahi KK, Liu C-C, Bhindi R, Figtree GA. Biological markers of oxidative stress: applications to cardiovascular research and practice. Redox biology 2013;1:483-91.

29. Lykkesfeldt J. Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. Clinica chimica acta 2007;380:50-58.

30. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative medicine and cellular longevity 2014;2014.

31. Negre-Salvayre A, Coatrieux C, Ingueneau C, Salvayre R. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. British journal of pharmacology 2008;153:6-20.

32. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Radical Biology and Medicine 1996;20:707-27.

33. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. Journal of Biological Chemistry 1997;272:20313-16.

34. Chevion M, Berenshtein E, Stadtman E. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radical Research 2000;33:S99-108.

35. Beal MF. Oxidatively modified proteins in aging and disease 1, 2. Free Radical Biology and Medicine 2002;32:797-803.

36. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clinica chimica acta 2003;329:23-38.

37. Pham-Huy LA, He H, Pham-Huy C. Free Radicals, Antioxidants in Disease and Health. International Journal of Biomedical Science : IJBS 2008;4:89-96.

38. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int j biomed sci 2008;4:89-96.

39. Bouayed J, Bohn T. Exogenous antioxidants—double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. Oxidative medicine and cellular longevity 2010;3:228-37.

40. Dimitrios B. Sources of natural phenolic antioxidants. Trends in food science & technology 2006;17:505-12.

41. Hopkins RZ. Nrf2 silencing for neuron maturation. Reactive oxygen species 2016;1:53-58.

42. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 2011;118:9-18.

43. Mandrup-Poulsen T, Nerup J, Reimers JI, Pociot F, Andersen HU, Karlsen A, Bjerre U, Bergholdt R. Cytokines and the endocrine system. I. The immunoendocrine network. European journal of endocrinology 1995;133:660-71.

44. Dinarello CA. Anti-inflammatory Agents: Present and Future. Cell 2010;140:935-50.

45. Kopf M, Bachmann MF, Marsland BJ. Averting inflammation by targeting the cytokine environment. Nature Reviews Drug Discovery 2010;9:703-18.

46. Dinarello CA. PRoinflammatory cytokines*. Chest 2000;118:503-08.

47. Zhang J-M, An J. Cytokines, Inflammation and Pain. International anesthesiology clinics 2007;45:27-37.

48. Kindt TJ, Goldsby RA, Osborne BA, Kuby J. Kuby immunology: Macmillan; 2007.

49. Goyal L, Bey A, Gupta ND, Sharma VK. Comparative evaluation of serum C-reactive protein levels in chronic and aggressive periodontitis patients and

association with periodontal disease severity. Contemporary clinical dentistry 2014;5:484-88.

50. Ridker PM. C-reactive protein a simple test to help predict risk of heart attack and stroke. Circulation 2003;108:e81-e85.

51. Negrulj R, Mooranian A, Al-Salami H. Potentials and limitations of bile acids in type 2 diabetes mellitus: applications of microencapsulation as a novel oral delivery system. Journal of Endocrinology and Diabetes Mellitus 2013;1:49-59.

52. Bruunsgaard H. Physical activity and modulation of systemic low-level inflammation. Journal of leukocyte biology 2005;78:819-35.

53. Dinarello CA. Historical Review of Cytokines. European journal of immunology 2007;37:S34-S45.

54. Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP. Multiplex bead array assays for detection of soluble cytokines: Comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry Part B: Clinical cytometry 2004;61b:35-39.

55. Martinon F. Signaling by ROS drives inflammasome activation. European journal of immunology 2010;40:616-19.

56. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: How are they linked? Free radical biology & medicine 2010;49:1603-16.

57. Hajjar DP, Gotto AM. Biological Relevance of Inflammation and Oxidative Stress in the Pathogenesis of Arterial Diseases. The american journal of pathology 2013;182:1474-81.

58. Khansari N, Shakiba Y, Mahmoudi M. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. Recent patents on inflammation & allergy drug discovery 2009;3:73-80.

59. Vîrgolici B, Mohora M, Găman L, LIXANDRU D, Manolescu B, COMAN A, STOIAN I. Relation Between Inflammation And Oxidative Stress Markers In Diabetic Foot Patients. inflammation 2008;6:13.

60. Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González Á, Esquivel-Chirino C, Durante-Montiel I, Sánchez-Rivera

G, Valadez-Vega C, Morales-González JA. Inflammation, oxidative stress, and obesity. International journal of molecular sciences 2011;12:3117-32.

61. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends in Immunology 2004;25:4-7.

62. Rubio-Perez JM, Morillas-Ruiz JM. A review: inflammatory process in Alzheimer's disease, role of cytokines. The Scientific World Journal 2012;2012.

63. Lall RK, Syed DN, Adhami VM, Khan MI, Mukhtar H. Dietary polyphenols in prevention and treatment of prostate cancer. International journal of molecular sciences 2015;16:3350-76.

64. Singh A, Holvoet S, Mercenier A. Dietary polyphenols in the prevention and treatment of allergic diseases. Clinical & experimental allergy 2011;41:1346-59.

65. Li A-N, Li S, Zhang Y-J, Xu X-R, Chen Y-M, Li H-B. Resources and Biological Activities of Natural Polyphenols. Nutrients 2014;6:6020-47.

66. Pérez-Jiménez J, Neveu V, Vos F, Scalbert A. Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. European journal of clinical nutrition 2010;64:S112-S20.

67. Vladimir-Knežević S, Blažeković B, Bival Štefan M, Babac M. Plant polyphenols as antioxidants influencing the human health. Phytochemicals as nutraceuticals–global approaches to their role in nutrition and health 2012:155-80.

68. Terao J, Murota K, Kawai Y. Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglyconein vivo. Food & function 2011;2:11-17.

69. Watson RR, Preedy VR, Zibadi S. Polyphenols in human health and disease: Academic press; 2013.

70. Lambert JD, Hong J, Yang G-y, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. The American journal of clinical nutrition 2005;81:284s-91s.

71. Salas-Salvadó J, Garcia-Arellano A, Estruch R, Marquez-Sandoval F, Corella D, Fiol M, Gómez-Gracia E, Vinoles E, Arós F, Herrera C. Components of the Mediterranean-type food pattern and serum inflammatory markers among

patients at high risk for cardiovascular disease. European journal of clinical nutrition 2008;62:651-59.

72. González-Gallego J, García-Mediavilla M, Sánchez-Campos S, Tuñón MJ. Fruit polyphenols, immunity and inflammation. British journal of nutrition 2010;104:S15-S27.

73. Veres B. Anti-inflammatory role of natural polyphenols and their degradation products: INTECH Open Access Publisher; 2012.

74. Quiñones M, Miguel M, Aleixandre A. Beneficial effects of polyphenols on cardiovascular disease. Pharmacological research 2013;68:125-31.

75. Gonzalez R, Ballester I, Lopez-Posadas R, Suárez M, Zarzuelo A, Martinez-Augustin O, Medina FSD. Effects of flavonoids and other polyphenols on inflammation. Critical reviews in food science and nutrition 2011;51:331-62.

76. Hu M-L. Dietary polyphenols as antioxidants and anticancer agents: more questions than answers. Chang gung med j 2011;34:449-60.

77. Bahadoran Z, Mirmiran P, Azizi F. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. J diabetes metab disord 2013;12:43.

78. Dall'Asta M, Bayle M, Neasta J, Scazzina F, Bruni R, Cros G, Del Rio D, Oiry C. Protection of pancreatic β-cell function by dietary polyphenols. Phytochemistry reviews:1-27.

79. Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R, Bapat P, Kwun I, Shen C-L. Novel insights of dietary polyphenols and obesity. The Journal of nutritional biochemistry 2014;25:1-18.

80. Tucakovic L, Colson N, Singh I. Relationship between Common Dietary Polyphenols and Obesity-Induced Inflammation. Food and public health 2015;5:84-91.

81. Derdemezis CS, Kiortsis DN, Tsimihodimos V, Petraki MP, Vezyraki P, Elisaf MS, Tselepis AD. Effect of plant polyphenols on adipokine secretion from human SGBS adipocytes. Biochemistry research international 2011;2011.

82. Shariati M, Sharifi E, Kaveh M. The effect of phoenix dactylifera (date-palm) pit powder on testosterone level and germ cells in adult male rats. ZUMS journal 2007;15:21-28.

83. Najafi MBH. Date seeds: a novel and inexpensive source of dietary fiber. International conference on food engineering and biotechnology, IPCBEF2011. p. 323-26.

84. Hamada J, Hashim I, Sharif F. Preliminary analysis and potential uses of date pits in foods. Food chemistry 2002;76:135-37.

85. Amin ES, Awad O, El Samad MA, Iskander M. Isolation of estrone from Moghat roots and from pollen grains of Egyptian date palm. Phytochemistry 1969;8:295-97.

86. Mohamed DA, Al-Okbi SY. In vivo evaluation of antioxidant and anti-inflammatory activity of different extracts of date fruits in adjuvant arthritis. Pol j food nutr sci 2004;13:397-402.

87. Care IoLARCo, Animals UoL, Resources NIoHDoR. Guide for the care and use of laboratory animals: National academies; 1985.

88. Reeves PG, Nielsen FH, Fahey Jr GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J nutr 1993;123:1939-51.

89. Roche. Cobas Integra 400 plus [cited 2015 27/August]. Available from: http://www.e-labdoc.roche.com/.

90. Roche. Cobas Integra 400 [cited 2015 20 December].

91. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J biol chem 1951;193:265-75.

92. Omaye ST, Tumball J, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. 1979.

93. Hatam LJ, Kayden HJ. A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. Journal of lipid research 1979;20:639-45.

94. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical biochemistry 1968;25:192-205.

95. Li X-Y, Chow CK. An improved method for the measurement of malondialdehyde in biological samples. Lipids 1994;29:73-75.

96. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz A-G, Ahn B-W, Shaltiel S, Stadtman ER. [49] Determination of carbonyl content in oxidatively modified proteins. Methods in enzymology 1990;186:464-78.

97. Milliplex KM. Rat Cytokine / Chemokine Magnetic Bead Panel. In: Millipore, editor. USA2015.

98. Al-Rasheed NM, Attia HA, Mohamad RA, Al-Rasheed NM, Al-Amin MA, AL-Onazi A. Aqueous Date Flesh or Pits Extract Attenuates Liver Fibrosis via Suppression of Hepatic Stellate Cell Activation and Reduction of Inflammatory Cytokines, Transforming Growth Factor-β1 and Angiogenic Markers in Carbon Tetrachloride-Intoxicated Rats. Evidence-based complementary and alternative medicine 2015;2015.

99. Drent M, Cobben N, Henderson R, Wouters E, van Dieijen-Visser M. Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung damage or inflammation. European respiratory journal 1996;9:1736-42.

100. Gowda S, Desai PB, Hull VV, Math AAK, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. The Pan african medical journal 2009;3:17.

101. Eid HH, Labib RM, Hamid NSA, Hamed MA, Ross SA. Hepatoprotective and antioxidant polyphenols from a standardized methanolic extract of the leaves of Liquidambar styraciflua L. Bulletin of Faculty of Pharmacy, Cairo University 2015;53:117-27.

102. Rahman K. Studies on free radicals, antioxidants, and co-factors. Clinical interventions in aging 2007;2:219-36.

103. Arnao MB, Cano A, Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food chemistry 2001;73:239-44.

104. Zitka O, Skalickova S, Gumulec J, Masarik M, Adam V, Hubalek J, Trnkova L, Kruseova J, Eckschlager T, Kizek R. Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients. Oncology letters 2012;4:1247-53.

105. Moskaug JØ, Carlsen H, Myhrstad MC, Blomhoff R. Polyphenols and glutathione synthesis regulation. The American journal of clinical nutrition 2005;81:277s-83s.

106. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biological chemistry 2009;390:191-214.

107. Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. The FASEB journal 1987;1:441-45.

108. Michels AJ, Frei B. Myths, artifacts, and fatal flaws: identifying limitations and opportunities in vitamin C research. Nutrients 2013;5:5161-92.

109. Traber MG, Stevens JF. Vitamins C and E: beneficial effects from a mechanistic perspective. Free radical biology and medicine 2011;51:1000-13.

110. Packer L, Fuchs J. Vitamin E in health and disease: Marcel dekker, Inc.; 1993.

111. Scholz R, Graham K, Gumpricht E, Reddy C. Mechanism of interaction of vitamin E and glutathione in the protection against membrane lipid peroxidation. Annals of the new york academy of sciences 1989;570:514-17.

112. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clinica chimica acta 2003;329:23-38.

113. Fedorova M, Kuleva N, Hoffmann R. Reversible and irreversible modifications of skeletal muscle proteins in a rat model of acute oxidative stress. Biochimica et biophysica acta (bba) - molecular basis of disease 2009;1792:1185-93.

114. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutrition, metabolism and cardiovascular diseases 2005;15:316-28.

115. Wu T, Rifai N, Roberts LJ, Willett WC, Rimm EB. Stability of measurements of biomarkers of oxidative stress in blood over 36 hours. Cancer Epidemiology biomarkers & prevention 2004;13:1399-402.

116. Meagher EA, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. Free Radical Biology and Medicine 2000;28:1745-50.

117. Hagfors L, Leanderson P, Sköldstam L, Andersson J, Johansson G. Antioxidant intake, plasma antioxidants and oxidative stress in a randomized, controlled, parallel, Mediterranean dietary intervention study on patients with rheumatoid arthritis. Nutrition journal 2003;2:1-11.

118. Hollman PC, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, Serafini M, Scalbert A, Sies H, Vidry S. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. The journal of nutrition 2011;141:989S-1009S.

119. Petersen A, Pedersen B. The role of IL-6 in mediating the anti inflammatory. J physiol pharmacol 2006;57:43-51.

120. Tuncer I, Özbek H, Topal C, Uygan I. The Serum Levels of IL-1\ beta, IL-6, IL-8 and TNF-\ alpha in Nonalcoholic Fatty Liver. Turkish journal of medical sciences 2003;33:381-86.

121. Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nature immunology 2015;16:448-57.

122. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei X-F, Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. Journal of clinical investigation 1998;101:311.

123. Camargo CA, Madden JF, Gao W, Selvan RS, Clavien P. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. Hepatology 1997;26:1513-20.

124. Cuevas A, Saavedra N, Salazar LA, Abdalla DSP. Modulation of Immune Function by Polyphenols: Possible Contribution of Epigenetic Factors. Nutrients 2013;5:2314-32.

125. González-Gallego J, García-Mediavilla MV, Sánchez-Campos S, Tuñón MJ. Fruit polyphenols, immunity and inflammation. British journal of nutrition 2010;104:s15-s27.

126. Tedgui A, Mallat Z. Anti-inflammatory mechanisms in the vascular wall. Circulation research 2001;88:877-87.

127. Rahmani AH, Al shabrmi FM, Allemailem KS, Aly SM, Khan MA. Implications of Green Tea and Its Constituents in the Prevention of Cancer via the Modulation of Cell Signalling Pathway. BioMed research international 2015;2015:925640.

128. Mühl H, Pfeilschifter J. Anti-inflammatory properties of pro-inflammatory interferon-γ. International Immunopharmacology 2003;3:1247-55.

129. Bhullar KS, Rupasinghe HPV. Polyphenols: Multipotent Therapeutic Agents in Neurodegenerative Diseases. Oxidative medicine and cellular longevity 2013;2013:891748.

130. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Current opinion in clinical nutrition and metabolic care 2010;13:541.